REMARKS

Claims 40-57 are pending in this application. Claims 1-39 have been cancelled without prejudice to Applicant's right to reintroduce the combinations of features claimed in the cancelled claims or to add additional claims covering the subject matter of the cancelled claims either later during prosecution of the present application or in applications related to the present one, like continuation, divisional and continuation-in-part applications.

New claims 40-57 have been added. Support for new claims 40-57 can be found *inter alia* on page 2 lines 20-24, page 3, lines 5-9, from page 3 line 19, to page 4 line 26, and in examples 1 and 2.

35 U.S.C. § 112 first paragraph

Independent claims 40, 46 and 52 are directed to methods for treating or preventing HCV infection in a patient.

In the Final Action of September 6, 2007, the Examiner observes that "the specification, while enabling for composition inhibiting HCV E2 binding to a cell (i.e. having neutralizing activity), does not reasonably provide enablement for composition for anti-HCV therapy comprising such neutralizing antibodies." (see Action, section 10 lines 2-4)

Applicant respectfully but strongly disagrees with the Examiner and draws the Examiner's attention to the following.

Applicant states that inhibition of E2 binding to a cell and neutralization activity are distinct properties of an antibody, which, as indicated in the specification, are not necessarily correlated to the each other.

Reference is made, in particular, to the exemplary passages of the specification on page 1 lines 27-30 ("*[h]ence the author has only evaluated and described the variable ability of different Fabs to inhibit the binding of protein E2 to the target cell, without demonstrating a correlation between this activity and the neutralizing activity of the sera*"), page 2 lines 20-23 ('*two of the assayed antibodies …. can neutralize the virus …, two other antibodies have no neutralizing activity …*") in view of page 16 lines 7-12 ("*two of the Fabs … do not neutralize VSV/HCV pseudotype infection … one of these two Fabs… has strong NOB activity…*"), page 17 lines 20-23 ("*..blocking of interaction between the virus and its cellular target seems unlikely to be a key factor in HCV neutralization. These data can explain at the molecular level the lack of correlation between NOB activity in the serum and protection from disease*), where underlining for emphasis purposes has been added. See also i) page 16 lines 13-19, ii) page 16, line 20 through page 17 line 17, and iii) table 2.

Additionally, it is clear from the above mentioned passages and from the remaining portions of the specification, that the wordings "neutralizing" "neutralizing antibody" and "neutralizing activity" as used in the specification refer to the neutralization of the HCV virus and not to neutralization of E2 binding to a cell which is instead indicated with wordings that

include the term "binding" such as "neutralization of binding activity", "NOB activity", or "inhibition of binding".

Accordingly, Applicant respectfully submits that the Examiner is mistaken when in section 10 of the Action the Examiner equates "inhibiting HCV E2 binding to a cell" to "having neutralizing activity", and based on this assumption incorrectly states that "the mere identification of an antibody as neutralizing antibody does not mean that those skilled in the art would be capable of using such in anti-HCV therapies."

Reference is also made to the enclosed declaration under 37 C.F.R. § 1.132, in particular to sections 6 to 13, wherein the distinction between inhibition/neutralization of binding and neutralization activity of an antibody and the common use of the above wording are further evidenced.

Applicant respectfully submits that mere identification of an antibody that neutralizes E2 binding to a cell is not indicative of anti-HCV therapeutic use of such antibody (see also page 16 lines 10-12 "*even antibodies inhibiting E2 binding may fail to prevent viral infection*"), while , on the other hand, identification of a neutralizing antibody, i.e. an antibody able to neutralize the HCV virus, is instead a very reliable indication of an anti-HCV use of such an antibody.

In support of the above statement, reference is made to the specification at page 3 lines 5-9 ("*[t]he identification of anti-E2 antibodies in the human Fabs format with a good neutralizing ability permits their large-scale production and use as a medication in anti-HCV treatment, or as a preventive agent in*

topical form to inhibit viral transmission to subjects at risk (couples with discordant HCV state, individuals subject to occupational exposure, etc.).)

As a further support, reference can also be made to the enclosed declaration under 37 C.F.R. § 1.132 and in particular to section 14 thereof, wherein the correlation between the ability to neutralize the virus, i.e. to render the virus inactive or ineffective, and the ability of treating and preventing a viral infection in a subject are further evidenced.

In the specification, the ability of the claimed antibodies and their functional fragments to neutralize the HCV virus *in vivo* is demonstrated and exemplified in a system of HCV pseudotypes. Reference can be made, in particular, to the specification at page 2 lines 13-19: ("*[t]he authors of the invention have also evaluated the neutralizing activity of various anti-E2 antibodies in a system of viral pseudotypes [VSV/HCV],… the method provides a direct measure of the in vivo neutralizing activity of anti-E2 antibodies…*";) page 16, lines 13-15: "*[t]wo other Fabs, e137 and e301, efficiently neutralize VSV/HCV at a concentration of 10 .mu.g/ml, while VSV pseudotypes bearing the VSV G envelope protein (VSV/G pseudotypes) are not affected*") and to figures 3A, 3B and 4, together with related portions of the specification, see in particular Example 2.

As a further supporting evidence, see the enclosed declaration under 37 C.F.R. § 1.132, in particular sections 13 and 15 of the same, wherein disclosure of the ability of the claimed antibodies and fragments thereof to neutralize the HCV virus *in vivo* in the specification is further evidenced.

Additional information concerning the use of the claimed antibodies or fragments thereof in treating or preventing HCV infection can be identified by the skilled person upon reading of the present application on the basis of the references cited and information known in the art. In this respect, reference is made to the enclosed declaration under 37 C.F.R. § 1.132, and in particular to its sections 15 and 16.

Therefore, Applicant submits that the present specification provides sufficient guidance to a skilled person to enable the use of the antibodies of claims 40, 46 and 52 without undue experimentation and that the specification thus fulfills the requirements of 35 U.S.C. 112 first paragraph.

35 U.S.C. § 102

In section 12 of the Action, the Examiner rejects previously presented claims 1, 27. 28, and 30-39 under 35 U.S.C. 102, as being anticipated by Burioni et al (Hepatology 28:810-14) (hereinafter Burioni).

In the interest of expediting prosecution of the present application, Applicant has cancelled the rejected claims without prejudice, thus rendering the above rejection moot.

New independent claims 40, 46 and 52 are directed to a method to treat or prevent HCV infection in a subject.

Applicant submits that Applicant was not able to find in Burioni a disclosure, teaching or suggestion related to antibodies that

are able to neutralize the HCV virus and/or to be used in treating or preventing HCV infection.

On the contrary, Applicant notices how in discussing the experimental results illustrated in his paper, Burioni first states that "*the results demonstrate that some human antibody specificities are able to neutralize the binding of HCV/E2 to its target cells*." (see page 812 second column lines 6-8) and then concludes that "*the correlation between NOB activity and true virus neutralization remains to be proven*" (see page 813 second column lines 9-11). Reference is also made to the enclosed declaration under 37 C.F.R. § 1.132, and in particular section 17 of such declaration.

Hence, Applicant submits that independent claims 40, 46 and 52 are novel over Burioni together with dependent claims 41-45, 47-51 and 53-57, at least by virtue or their dependency on said independent claims.

35 U.S.C. § 103

In section 15 of the Action, the Examiner rejects previously presented claims 1-3, 27 and 28, 30 and 39 under 35 U.S.C. 103 as being unpatentable over Burioni, in view of the teachings of Poul (Immunotechnology 1:189-96) (herein after Poul) and Foung et al (US 7,091,324), hereinafter (Foul)

In the interest of expediting prosecution of the present application, Applicant has cancelled the rejected claims without prejudice, thus rendering the above rejection moot.

New independent claims 40, 46 and 52 are directed to a method to treat or prevent HCV infection in a subject.

In rejecting the previous claims and addressing Applicant's arguments, the Examiner stated that "the Applicant must show that the claimed invention has unexpected results over the "closest prior art…" (see Action section 15, lines 7-8)

Applicant has already shown in the previous section that Burioni, considered by the Examiner to be "the closest prior art", does not disclose, teach or suggest antibodies able to neutralize HCV virus and/or a related use in treatment or prevention of HCV infection.

In addition to that, Applicant was not able to find in the other references cited by the Examiner such teaching or a teaching that would make that result reasonably expected for antibodies having an NOB activity and in particular for the two fragments e137 and e301. On the contrary, "the closest prior art" Burioni, clearly indicates that the "*correlation between NOB activity and true virus neutralization remains to be proven*." (see page 813 second column lines 9-11).

In this connection, reference can also be made to the declaration attached hereto as **Exhibit A** under 37 C.F.R. § 1.132 and in particular to sections 17-118 of the same, wherein the above statements are further evidenced.

Therefore, Applicant submits that any person skilled in the art, faced with the prior art cited by the Examiner, would not have any reasonable expectation on the ability of e137 and e301 to

neutralize HCV *in vivo* and to be usable in treating or preventing HCV infection over the cited art.

Conclusion

In view of the above, Applicant submits that new independent claims 40, 46 and 52 are patentable over the cited art together with dependent claims 41-45, 47-51 and 53-57 also patentable at least by virtue of their dependency on said independent claims.

Should the Examiner disagree with the Applicant, the Examiner is respectfully invited to indicate the passages in the cited art that in combination with Burioni would make that result reasonably expected.

If a telephone interview would be of assistance in advancing the prosecution of the subject application, Applicants' undersigned attorney invites the Examiner to telephone him at the number provided below. If any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 50-1891.

Respectfully submitted,

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EXHIBIT A

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Roberto Burioni	Examiner: Lucas, Zachariah
Serial No.: 10/502,307	Art Unit: 1648
Filed: July 22, 2004	Our Ref: 937-PCT-US
For: "HUMAN MONOCLONAL ANTIBODY FAB FRAGMENTS DIRECTED AGAINST HCV E2 GLYCOPROTEIN AND ENDOWED WITH IN VITRO NEUTRALIZING ACTIVITY"	Re: ***Amendment and Response***

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I,Massimo Clementi, declare and say:

1. I am Massimo Clementi, MD, Full Professor of Microbiology and Virology at the Medical School of Università San Raffaele, Milan, Director of the Microbiology and Virology Laboratory of the San Raffaele Hospital, Milan, Italy

2. I graduated in Medicine from the University of Ancona and received a Specialty degree in the field of Infectious Diseases from the Università of Rome, Italy

3. I have been working in the field of Virology since about 1976 I have spent the past 30 years principally in research regarding the virus-host interplay. I have directed since then laboratories working on programs aimed to elucidate the nature of the pressure on viral human pathogens by the immune system.

4. I am a co-inventor of several issued U. S. patents. I have co-authored hundreds of scientific papers in this field and have given talks on the subject at over 600 conferences.

5. I am familiar with the above-identified application, Burioni et al (Hepatology 28:810-14) (hereinafter Burioni), Habersetzer et al (Virology 249:32-41) (hereinafter Habersetzer), Poul (Immunotechnology 1:189-96) (herein after Poul) and Foung et al (US 7,091,324), hereinafter (Foul) and have read the Office Actions of March 02, 2007 and September 6, 2007.

6. I respectfully submit that the Examiner is mistaken when the Examiner equates neutralization of binding (NOB) to neutralization activity of the antibodies described in the above identified application.

7. As used in the relevant technical field, the wording NOB refers to the activity of an antibody (or another molecule) in inhibiting the binding of a viral protein, such as the E2 envelope protein of HCV, to a cellular target. The wording neutralization activity, which generally indicates virus neutralizing activity refers instead to the activity of an antibody (or another molecule) in blocking viral infection in an *in vitro* setting. It is well known from several studies both in viruses causing an acute infection (influenza) and viruses causing persistent infection (HIV) with a virus-host interplay similar to the one present in HCV infection that inhibition of the binding between the virus envelope protein and the cellular target does not correspond to neutralization or neutralizing activity. For these reason NOB is completely different from neutralization activity, as would be understood by a skilled person. –

8. In the context of the present patent application the wording "neutralizing activity" is consistently used to indicate virus neutralizing activity in an in vitro pseudovirus setting, and not NOB, as clearly showed by Table 2 of the above identified application, where the lack of correlation between NOB activity and pseudovirus neutralizing activity is shown.

9. At the time the invention was made, evidence for a lack of correlation between NOB and neutralizing activity was available for HCV (see e.g. Matsura et al. (Virology 286, 263-275 (2001)) herein enclosed with particular reference to page 269 col. 2 lines 13-31). In addition to that, evidences regarding several other other viral models clearly indicated that "*inhibition of virus attachment to cells by antibodies has been reported only rarely as the major contribution to infectivity neutralization*" (see Knossow et al. (Virology 302, 294–298 (2002)) enclosed herewith with particular reference to page 296 column 1 lines 7-11) The same was known as true in persistently replicating viruses causing persistent infections such as HIV (see e.g. Barbas et al, PNAS 89, , (1992) enclosed herewith page 9339 column 2 lines 29-32 and 9342 starting from column 1 line 17) and for human monoclonal Fab fragments, demonstrating how antibodies that block interaction between the viral protein and the cellular target are usually not able to neutralize infection.

10. NOB and virus neutralization activity are measured by completely different tests in HCV and in several other viral models. Any expert with a knowledge of the state of the art could at the moment of the invention understand that a test related to NOB could not be indicative of neutralizing activity in HCV or other viral models and *vice versa*

11. In particular, the NOB activity of an antibody is measured by incubating the viral protein to be bound, which in HCV is the E2 envelope protein, (produced in a recombinant setting and in a soluble truncated form) with the purified antibody at different concentrations. This mixture is then put in contact with a target cell population, and this cell population is then stained, directly or indirectly, with an anti-HCV/E2 mouse monoclonal antibody. This staining is measured and it indirectly measures the binding of the HCV/E2 glycoprotein to the target cell, thus allowing the evaluation of the antibody-mediated inhibition of binding. This measure is not appropriately called "neutralization of binding", as "inhibition of binding" would be more appropriate.

12. The neutralizing activity of an antibody is measured instead in vitro by incubating a known amount of infecting virus with increasing concentrations of purified antibody. The mixture is then used to infect susceptible cells, and the concentration of antibodies inhibiting 50% of 90% of the infection is extrapolated and named IC50 or IC90, respectively. When there are not suitable in vivo virus replication experimental systems, or when this procedure is dangerous due to the nature of the virus, it is possible to perform the test with pseudoviruses. A pseudovirus is an artificial virus bearing in its envelope the glycoproteins of the virus, which is impossible or difficult to replicate (pseudotyped virus), and includes on the viral genome a reporter gene that can be easily demonstrated inside a susceptible cell once the pseudovirus has entered it (typically a green fuorescent protein). Due to the fact that the glycoproteins in the pseudovirus envelope are derived from the pseudotyped virus, the entry mechanisms are derived from the pseudotyped virus itself, and this can be used to study neutralization activity of an antibody against envelope proteins of the pseudotyped virus when replicating this virus is impossible or undesirable

13. While binding inhibition tests, such as NOB, are not considered predictive at all of neutralizing activity of a compound, pseudoviruses are considered the most reliable approach for the measurement of neutralizing activity of a virus that is impossible to replicate in vitro. This has been proven true also for HCV once a replication system has become available, as antibodies (such as e137) endowed with neutralizing activity against pseudoviruses showed a strong neutralization activity also against HCV in cell culture settings, as illustrated in Perotti et al (in Journal of Virology, Jan. 2008, p. 1047–1052 Vol. 82, No. 2 also enclosed herewith. The lack of correlation between the NOB activity and virus neutralization can be explained, as an example, by the fact that the NOB activity is measured using a ricombinant and soluble monomeric protein of the virus and to the skilled person eyes, it is not surprising that antibodies binding the viral protein in the monomeric format and showing a strong NOB activity, are not able

to neutralize the virus infectivity in vitro. Furthermore the available protein surface can be different on the virion surface because of the heterodimeric interaction of the viral protein leading to an altered accessibility of the regions bound by antibodies. Therefore the NOB activity is not predictive of neutralizing activity because an antibody may not bind the heterodimeric protein (as the protein really present on the surface of the virus) at the same extent of the monomeric protein.

14. When considering the antiviral properties of an antibody, the in vitro neutralization activity against the viral target of the antibody is by far the most important activity, if not the only one, indicating that the compound, showing a neutralizing activity at a concentration that can be reached in vivo, is a powerful tool in clinical setting for the therapy and the prevention of a viral infection. This indicates a usefulness of the compound in the therapy or the prevention of a viral infection. This has been proven in all experimental and clinical setting. Neutralizing activity in the serum is often a sign of protection against reinfection by a given virus, and, in particular, presence of pseudovirus neutralizing activity in the sera of acute HCV patients has been shown to be correlated to a better prognosis

15. The pseudovirus neutralization data contained in this patent application, showing for the first time these moleculesbeing endowed with a very strong neutralizing activity at low concentration very effective concentrations that can be reached in vivo, are clearly identifying these antibodies at the skilled person's eyes, as the first and only candidates at the patent filing time as useful for treatment and prevention of HCV infection, being neutralizing and thus being able of blocking or contrasting viral replication and viral spreading to non-infected cells when administered to a patient .

16. For all of the above reasons, one skilled in the art upon reading of the above application would be enabled to perform the methods of claims 40 to 57 and would reasonably conclude that the inventor had possession of such methods at the time the invention was made.

17. Further, in view of the above and upon review of Burioni, Habersetzer, Poul and Foung, the declarant believes these references do not disclose, teach or suggest neutralization of the virus and that ability to neutralize virus to the extent shown in application is unexpected. This is particularly true as existence of HCV neutralizing antibody clones in the human anti-HCV antibody repertoire was not expected, considering the fact that recovering from an acute HCV infection provides the recovered patients with a strong humoral immunity that does not provide protections. This patent application shows that neutralizing antibody clones are existing in the repertoire of infected patients, but they are coexisting with other, more frequent clones, that have not such an effect. The neutralizing effect of the human antibody clones, as shown in Table 2 of the above identified application can not be foreseen by the evaluation of NOB activity, being the human antibody from the repertoire of this patient endowed with the highest NOB activity actually completely devoid of neutralizing activity, and eventually endowed with facilitating activity for viral infection

18. In particular, given that Burioni and the other art only show the sequence and the NOB activity of e137 and e310, in NO WAY a skilled person could expect such anti HCV antibody clones to have a neutralizing activity.

19. I declare further that all statements made herein of my own knowledge are true; that all statements made herein on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false

statements may jeopardize the validity of this application or any patents issuing thereon.

Date: February 26th, 2008

Massimo Clementi

Virology 286, 263–275 (2001)
doi:10.1006/viro.2001.0971, available online at http://www.idealibrary.com on IDEAL®

Characterization of Pseudotype VSV Possessing HCV Envelope Proteins

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Received January 22, 2001; returned to author for revision March 30, 2001; accepted April 26, 2001

The genome of hepatitis C virus (HCV) encodes two envelope glycoproteins (E1 and E2), which are thought to be responsible for receptor binding and membrane fusion resulting in virus penetration. To investigate cell surface determinants important for HCV infection, we used a recombinant vesicular stomatitis virus (VSV) in which the glycoprotein gene was replaced with a reporter gene encoding green fluorescent protein (GFP) and produced HCV-VSV pseudotypes possessing chimeric HCV E1 or E2 glycoproteins, either individually or together. The infectivity of the pseudotypes was determined by quantifying the number of cells expressing the GFP reporter gene. Pseudotypes that contained both of the chimeric E1 and E2 proteins exhibited 10–20 times higher infectivity on HepG2 cells than the viruses possessing either of the glycoproteins individually. These results indicated that both E1 and E2 envelope proteins are required for maximal infection by HCV. The infectivity of the pseudotype virus was not neutralized by anti-VSV polyclonal antibodies. Bovine lactoferrin specifically inhibited the infection of the pseudotype virus. Treatment of HepG2 cells with Pronase, heparinase, and heparitinase but not with phospholipase C and sodium periodate reduced the infectivity. Therefore, cell surface proteins and some glycosaminoglycans play an important role in binding or entry of HCV into susceptible cells. The pseudotype VSV possessing the chimeric HCV glycoproteins might offer an efficient tool for future research on cellular receptors for HCV and for the development of prophylactics and therapeutics for hepatitis C. © 2001 Academic Press

Key Words: hepatitis C virus; envelope proteins; pseudotype virus; infection.

INTRODUCTION

Hepatitis C virus (HCV) is the most important causative agent of posttransfusion and sporadic non-A non-B hepatitis, infecting more than 200 million people worldwide (Houghton, 1996). HCV infection becomes chronic in most cases and may eventually result in hepatitis, liver cirrhosis, and hepatocellular carcinoma (Alter *et al.*, 1992; Saito *et al.*, 1990). This high percentage of chronicity after HCV infection can be explained by (i) HCV escape from the host protective immune responses by mutating its amino acid sequences (Kato *et al.*, 1993; Weiner *et al.*, 1992) or by (ii) the failure of HCV infection to induce protective immune responses (Farci *et al.*, 1992; Weiner *et al.*, 1995). The HCV genome encodes two envelope glycoproteins (E1 and E2), which are derived from a precursor polyprotein after the polyprotein is processed by signal peptidase. The two glycoproteins form a heterodimer complex in the endoplasmic reticulum (ER) (Deleersnyder *et al.*, 1997; Dubuisson *et al.*, 1994, 2000; Dubuisson, 2000; Grakoui *et al.*, 1993; Matsuura *et al.*, 1994; Ralston *et al.*, 1993; Selby *et al.*, 1994), which is the site where virus budding is thought to occur.

Although some cell lines have been shown to support HCV replication, which was only detectable by nested PCR (Ito *et al.*, 1996; Mizutani *et al.*, 1996; Shimizu *et al.*, 1992), the lack of a conventional cell culture system for HCV has greatly limited studies of the infection mechanisms of HCV and the assessment of protective antibody responses to HCV. Rosa and colleagues (1996) reported that a truncated form of the HCV E2 protein (type 1a) can bind to Molt-4 cells and that the interaction could be inhibited by antibody. This assay was named the neutralization of binding (NOB). We have shown that the appearance and maintenance of high titers of NOB antibodies are accompanied by clinical resolution of liver disease and virus clearance (Ishii *et al.*, 1998). More recently it was reported that human CD81 (hCD81) could be bound by a truncated, soluble form of the E2 protein (Pileri *et al.*, 1998), suggesting that hCD81 may act as a receptor for HCV on the cell surface; however, it is not known whether hCD81 serves as a functional receptor and can lead to a productive HCV infection.

One of the ways to overcome the lack of a conventional cell culture system for HCV is to generate pseudotype viruses that have HCV envelope proteins on

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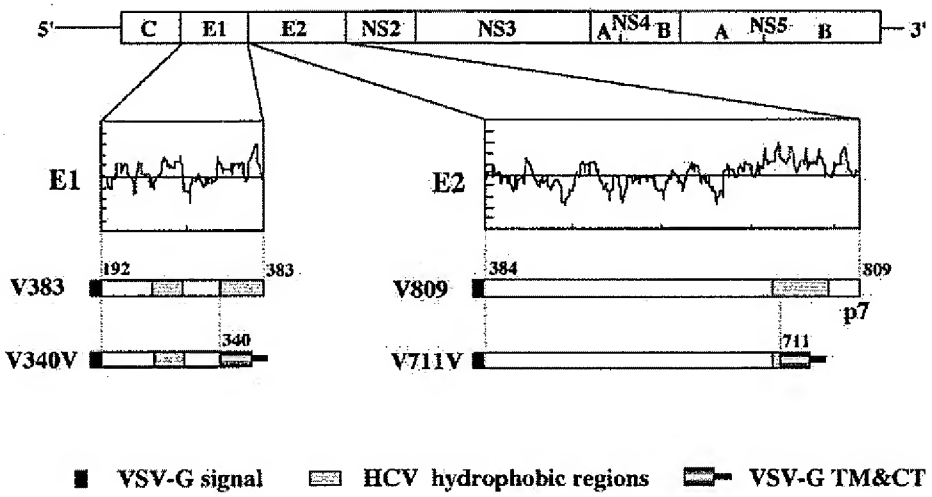

FIG. 1. Chimeric HCV envelope genes used in this study. The gene structure of HCV is shown at the top. Hydrophobic profiles of amino acid sequences of HCV E1 and E2 proteins are shown in the middle. V340V and V711V are chimeric proteins, which have deletions in C-terminus and that possess the transmembrane and cytoplasmic tail of VSV-G protein. V383 and V809 are full-length cDNA clones. Black bars in the N-terminus indicate the signal sequence of VSV-G protein. Gray bars are the putative transmembrane regions of the HCV envelope proteins.

the surface of another virus (Rubin, 1965). Vesicular stomatitis virus (VSV) can be efficiently propagated in many animal cells and readily forms pseudotypes with the envelope proteins from many different viruses. Recently, systems to recover recombinant Rhabdoviruses, specifically rabies virus and VSV, from cDNA clones have become available (Lawson *et al.*, 1995; Schnell *et al.*, 1994; Whelan *et al.*, 1995). In addition, it has been demonstrated that foreign glycoproteins can be incorporated efficiently into recombinant rhabdovirus envelopes (Mebatsion *et al.*, 1996; Schnell *et al.*, 1996). One approach that has been used to study the entry mechanism of a number of different viruses that are either difficult to grow or that require high-level containment facilities utilizes a novel recombinant VSV (VSVΔG*) that encodes the green fluorescent protein (GFP) gene instead of the glycoprotein (G) gene. Transient expression of foreign envelope glycoproteins in VSVΔG*-infected cells resulted in the release of infectious pseudotype viruses which were subsequently used to examine the cell entry properties conferred by the foreign viral proteins (Okuma *et al.*, 2001; Takada *et al.*, 1997; Tatsuo *et al.*, 2000).

To examine the role of the two HCV envelope proteins in cell infection, we produced pseudotype VSVs that had modified HCV envelope proteins, either individually or together, instead of the VSV G glycoprotein. Both of the HCV E1 and E2 proteins have C-terminal retention signals and these proteins are normally retained in the ER (Cocquerel *et al.*, 1998, 1999). To incorporate the HCV envelope proteins into VSV virions, the envelope proteins must be expressed on the cell surface. Therefore, chimeric proteins containing the signal sequence, transmembrane domain, and cytoplasmic tail of VSV G glycoprotein and the ectodomain of HCV E1 or E2 protein were generated (Takikawa *et al.*, 2000). Incorporation of both of the chimeric E1 and E2 proteins into virions was required to confer maximal infectivity on the pseudotype virions. Among the cell lines examined, HepG2 cells exhibited the highest susceptibility to the pseudotype VSV. Chemical modification analyses suggest that protein molecules and glycosaminoglycans on HepG2 cells may play an important role in the infection of HCV.

RESULTS

Characterization of CHO cell lines expressing the chimeric HCV envelope proteins

To generate pseudotype VSVs bearing HCV envelope proteins, it was necessary to express both E1 and E2 on the cell surface, since VSV assembles and buds from the plasma membrane. CHO cell lines constitutively expressing chimeric E1 and E2 proteins, either individually (CHOE1 and CHOE2) or together (CHOE1E2), were established using chimeric cDNA constructs encoding the ectodomains of E1 or E2 joined to the transmembrane and cytoplasmic domains of VSV-G protein (Takikawa *et al.*, 2000) (Fig. 1). To confirm that the chimeric proteins were expressed on the cell surface, the CHO cell lines were analyzed by flow cytometry using monoclonal antibodies against E1 or E2 protein. The appropriate chimeric protein was detected on the cell surface of each of the E1 or E2 CHO cell lines, and both proteins were present on the CHOE1E2 cell line (Fig. 2A). To examine the processing of the chimeric E1 and E2 proteins, the CHOE1E2 cell line was pulse labeled for 15 min with [^{35}S]methionine and cysteine and chased for 4 h and the proteins were immunoprecipitated by E1- or E2-specific monoclonal antibodies (Fig. 2B, left). The chimeric E1 protein migrated as a doublet with molecular weights of 34 and 36 kDa soon after labeling, which then shifted

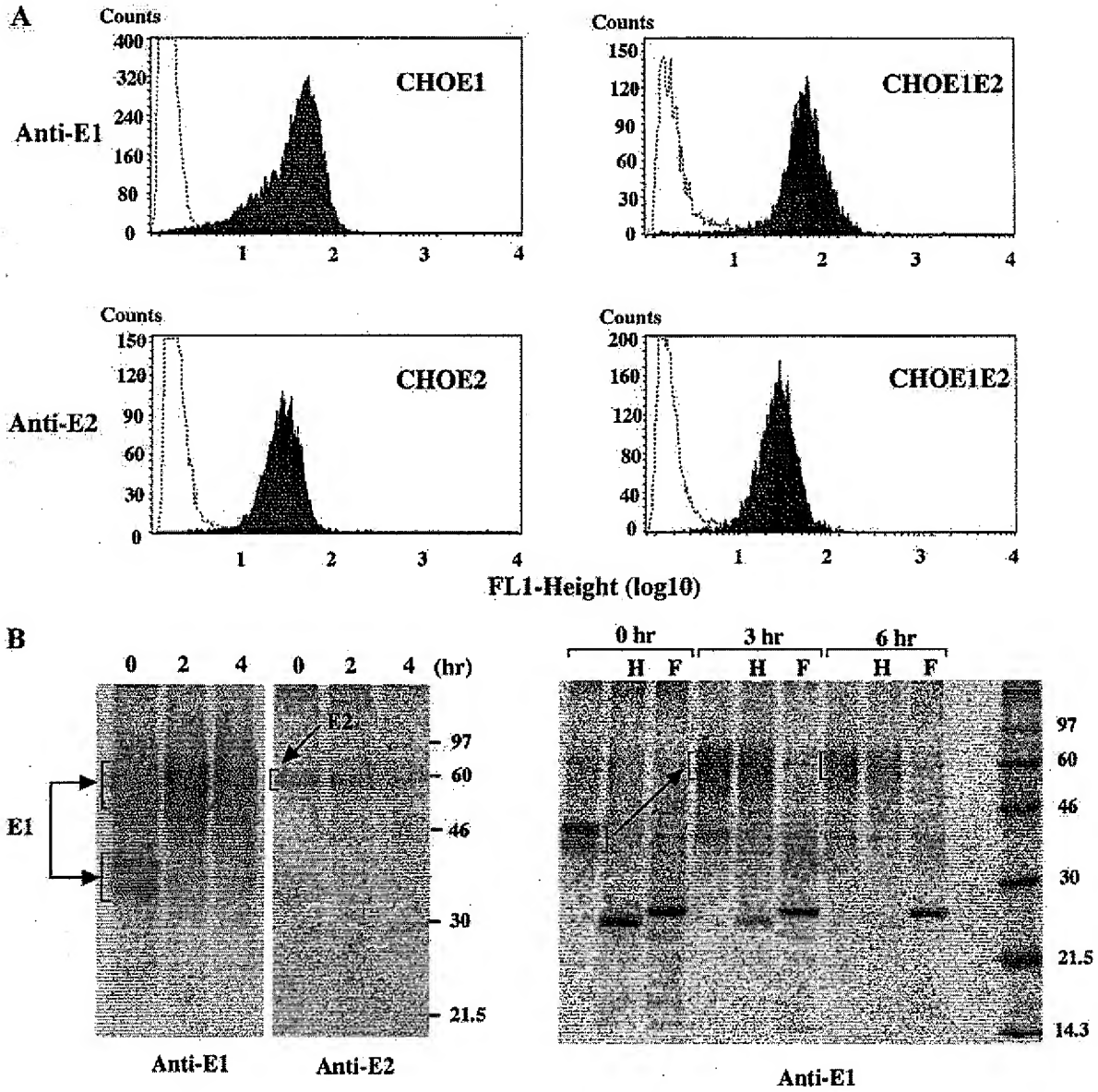

FIG. 2. (A) Cell surface expression of chimeric HCV envelope proteins on the CHO cell lines. CHO cell lines were immunostained with anti-E1 or E2 monoclonal antibody and FITC-conjugated goat anti-mouse IgG and analyzed by flow cytometry. The level of cell surface expression is indicated by the shift of the solid histogram to the right from the control histogram (open, dashed line) in which cells were stained with the FITC-conjugated antibody only. Axes: FL1-Height, fluorescence intensity; Counts, number of cells. (B) Left, pulse-chase analyses of CHO cell line. CHOE1E2 cell line was labeled for 15 min with Tran ^{35}S-label and chased for 2 and 4 h. The cell lysates were immunoprecipitated with either the E1 or E2 monoclonal antibodies. Right, CHOE1E2 cell line was labeled for 15 min with Tran ^{35}S-label and chased for 3 and 6 h. The cell lysates were immunoprecipitated with anti-E1 monoclonal antibody and the immunoprecipitates were digested with Endo H (H) or PNGase F (F). The positions of the chimeric HCV envelope proteins are indicated by the arrows. Molecular weight markers are shown on the right.

into diffuse bands of 50–60 kDa after 2 h chase in both the CHOE1 (not shown) and the CHOE1E2 cells. In contrast, the size of the chimeric E2 protein in both the CHOE2 (not shown) and the CHOE1E2 lines was 60 kDa, and this did not change during the chase period. Under the conditions used for immunoprecipitation, we could not detect any heterodimer formation between the chimeric E1 and E2 proteins in the CHOE1E2 cell line. This might be a consequence of weak ectodomain interactions of the chimeric HCV envelope proteins that are not maintained in the detergents used for lysis. To examine the oligosaccharides on the chimeric E1 protein, the CHOE1E2 cell line was pulse labeled with radioactive methionine and cysteine and chased for 6 h, and the chimeric protein was immunoprecipitated with an anti-E1 monoclonal antibody. The immunoprecipitate was digested with endo-β-*N*-acetylglucosaminidase H (Endo H) or peptide-*N*-glycosidase F (PNGase F) (Fig. 2B, right). After a 6-h chase, the chimeric E1 protein was resistant to Endo H treatment, suggesting that 60-kDa species

resulted from the addition of complex-type carbohydrates. In contrast, the E2 protein did not appear to acquire complex oligosaccharides and migrated at the same molecular weight from the time of synthesis until at least 4-h postchase.

Although the exact molecular characteristics of HCV envelope proteins in the virion are not known yet, the carbohydrates of the envelope proteins on the virions are probably processed into complex type during transport to the cell surface, even if the virions bud into the ER lumen.

Production of pseudotype VSVs

To determine whether the chimeric proteins could be incorporated into VSV particles, we infected the two CHO cell lines expressing each of the chimeric envelope proteins individually, or the cell line expressing E1 and E2 together, with VSVΔG*-G, which consists of ΔG* virions complemented in trans with VSV-G protein (Fig. 3A). After 16 h the infected cell supernatants were collected and the pseudotyped viruses were purified by centrifugation through sucrose density gradients and analyzed by immunoblotting (Fig. 3B). As controls, VSVΔG*-G and VSVΔG* were produced by infecting CHO cells transiently expressing VSV-G protein, or the parental CHO cells, with VSVΔG*-G, respectively. The VSV structural proteins N, P, and M (matrix protein) were present in all of the purified virions. VSV-G protein was only detected in VSVΔG*-G (lane 1) but not in VSVΔG* negative control (lane 2) nor in the HCV pseudotype viruses (lanes 3–5). Virions produced in cells expressing the chimeric HCV envelope proteins contained either E1 or E2 (lanes 3 and 4) or both of the chimeric envelope proteins (lane 5), indicating that both proteins were competent for assembly into VSV particles.

Infectivity of HCV pseudotype VSVs

To examine whether the pseudotype viruses possessing HCV envelope proteins could rescue the infectivity of VSV lacking G protein, we incubated HepG2 cells with supernatants containing the pseudotype viruses. The infection was then determined by examining the cells for GFP expression using fluorescence microscopy (Fig. 4). The titer of VSV-E1E2 was about 10–20 times higher ($\sim 4 \times 10^5$ IU/ml) than that of VSV-E1, VSV-E2, or VSVΔG* ($< 2 \times 10^4$ IU/ml), indicating that both of the chimeric proteins were required for maximal infectivity. We next examined the susceptibility of various cell lines to VSV-E1E2, VSVΔG*, and VSVΔG*-G (Table 1). HepG2 cells exhibited the highest susceptibility followed by COS7, CV-1, 293T, and Huh7 cells. The other cell lines, including the NIH3T3 cell line expressing hCD81, showed no detectable susceptibility to VSV-E1E2.

Neutralization of the pseudotype virus

To determine whether the infectivity of VSV-E1E2 was HCV specific, we examined whether the pseudotypes could be neutralized by a polyclonal antibody against VSV. As shown in Fig. 5, the anti-VSV antibody completely neutralized VSVΔG*-G, whereas no neutralization was observed with the VSV-E1E2 pseudotypes. These results suggest that infectivity of the VSV-E1E2 was not due to residual inoculum nor due to adventitious incorporation of VSV-G protein into the pseudotype virions. However, no neutralization was observed with the sera from a chimpanzee immunized with HCV E1 and E2 proteins, nor with sera from patients possessing neutralization of binding antibodies, nor with mouse monoclonal antibodies recognizing HCV envelope proteins.

Effects of bovine lactoferrin and Suramin on pseudotype infection

Previously it was shown that bovine lactoferrin, a milk protein belonging to the iron transporter family, prevented HCV infection in human hepatocytes (Ikeda *et al.*, 1988). In another study, Suramin, which is a polysulphonate pharmaceutical, blocked binding of HCV to human hepatocytes (Garson *et al.*, 1999). To examine the effect of bovine lactoferrin or Suramin on the infection of the pseudotype virus, VSV-E1E2 and VSVΔG*-G were preincubated with the compounds and then the inoculum was added to HepG2 cells (Fig. 6). Pretreatment with bovine lactoferrin reduced the infectivity of VSV-E1E2 in a dose-dependent manner, whereas no effect was observed on the infectivity of VSVΔG*-G. Similarly, preincubation with Suramin reduced the infectivity of VSV-E1E2, but the infectivity of VSVΔG*-G was also inhibited at the higher concentrations.

Effects of chemical modifications of HepG2 cells on infectivity of the pseudotype VSV

To obtain information on the biological characteristics of cellular receptors for HCV, we examined the infectivity of the pseudotype VSV-E1E2 on chemically modified HepG2 cells. HepG2 cells were treated with various concentrations of protease, sodium periodate, or phospholipase C and then the treated cells were infected with 2.5×10^3 IU of the VSV-E1E2 or VSVΔG*-G. The effects of the chemical modifications on infectivity were evaluated by counting the number of GFP-positive (infected) cells using fluorescence microscopy (Fig. 7A). When HepG2 cells were treated with Pronase, the infectivity of the VSV-E1E2 was markedly reduced compared to the infectivity of VSVΔG*-G. However, treatment with phospholipase C or sodium periodate did not inhibit the infection with either of the viruses. These results suggest that protein molecule(s) on cell surface plays an important role in infection of VSV-E1E2.

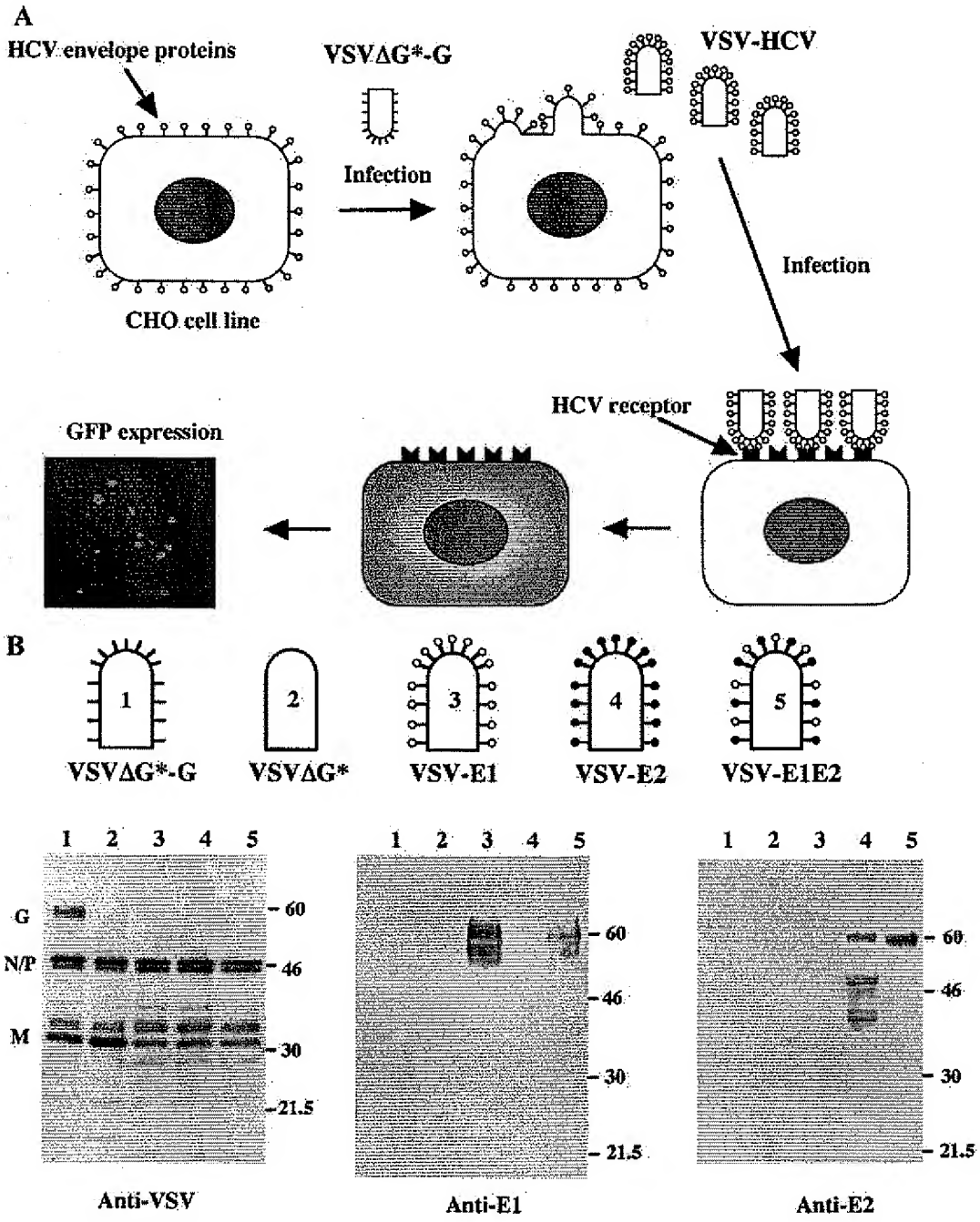

FIG. 3. (A) Generation of pseudotype VSVs in CHO cell lines constitutively expressing the chimeric HCV envelope proteins and infectivity assays of the pseudotype VSVs. (B) Western blot analyses of the pseudotype VSVs possessing HCV chimeric envelope proteins. Schematic representation of the pseudotype VSVs is shown at the top. (1) VSVΔG*-G is complemented with the VSV G protein, but the genome contains the GFP gene instead of the VSV G gene. (2) VSVΔG* has no envelope protein and was prepared in the parental CHO cells infected with VSVΔG*-G. (3) VSV-E1, (4) VSV-E2, and (5) VSV-E1E2 were recovered by inoculation of VSVΔG*-G into CHOE1, CHOE2, and CHOE1E2, respectively. The purified virions were analyzed with Western blots using an anti-VSV polyclonal antibody, an anti-E1 or an anti-E2 monoclonal antibody. G, N, P, and M are the glycoprotein, nucleocapsid protein, phosphoprotein, and matrix protein of VSV, respectively.

Glycosaminoglycans (GAGs) are unbranched polysaccharides ubiquitously present on cell surfaces and have been shown to be important in the cell surface binding of a number of bacteria, parasites, and viruses (Rostand and Esko, 1997). In dengue virus, for example, a highly sulfated heparan sulfate has been shown to effectively prevent the infection of target cells (Chen *et al.*, 1997). To examine the role of GAGs on the infectivity of the pseudotype virus, HepG2 cells were treated with several GAG lyases. Treatment of HepG2 cells with heparinase or heparitinase reduced the infectivity of the VSV-E1E2, whereas no reduction in infectivity was observed after the cells were treated with keratanase, hyaluronidase, or chondroitinase (Fig. 7B).

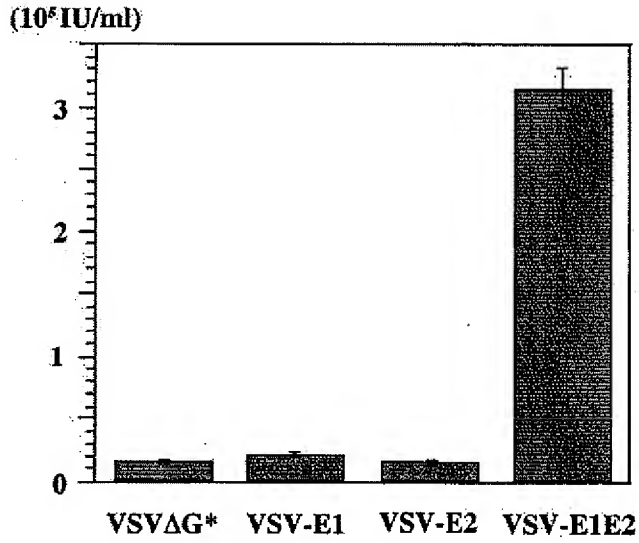

FIG. 4. Infectivity of the pseudotype VSVs on HepG2 cells. The pseudotype VSVs produced in the CHO cell lines constitutively expressing the chimeric HCV envelope proteins were added to HepG2 cells and infectivity of the pseudotype viruses was determined by quantifying the number of GFP expressing cells. The results shown are from three independent assays, with the error bars representing the standard deviation (SD).

DISCUSSION

A high risk of chronicity is the major concern of HCV infection. Chronic infection of HCV often leads to liver cirrhosis and hepatocellular carcinoma. The elimination of HCV from chronic hepatitis patients is of utmost importance. However, the complete removal of HCV has not been accomplished even at the experimental level. Furthermore, the study of the infection mechanism of HCV has been hampered by the lack of an efficient cell culture-based infection-neutralization assay.

To overcome these problems, we produced pseudotyped VSVs possessing chimeric HCV glycoproteins and then examined the infectivities of the pseudotypes by a simple fluorescence assay based on the expression of GFP from the pseudotyped viral genome. To assess the neutralization activity of HCV-specific antibodies, a NOB assay has been developed which measures the inhibition of binding of the purified E2 protein to hCD81 (Ishii *et al.*, 1998; Rosa *et al.*, 1996). However, the correlation between the NOB activity and true virus neutralization activity remains to be proven. We examined the sera from the immunized chimpanzees as well as the sera from patients possessing NOB antibodies; however, none of these had neutralization activity against the pseudotyped VSVs. These results suggest that some antibodies, which can inhibit E2 binding, may not prevent HCV infection. The use of the pseudotype virus allows the examination of multiple steps of infection, including binding both to the host receptor and to the membrane fusion. It therefore might be more relevant to examine the native functions of HCV envelope proteins using pseudotype VSV bearing both E1 and E2 proteins rather than the simple binding assay of the purified E2 protein to hCD81. Although hCD81 was sug-

TABLE 1

Infectivities of Pseudotype VSVs for Different Cell Lines

		Infectious units/ml ($\times 10^3$)		
Species	Cell line	VSV-E1E2	VSVΔG*-G	VSVΔG*
Human	HepG2	314.0	347.0	15.0
	Huh7	29.4	13.9	8.0
	FLC4	2.2	472.0	0
	293T	61.0	497.0	7.7
	HeLa	0.3	26.5	0
Monkey	COS7	121.8	10.8	9.0
	CV-1	67.0	173.0	14.0
	MA104	4.2	45.1	0
Swine	CPK	2.2	194.7	0
Bovine	MDBK	0	14.3	0
Canine	MDCK	2.0	1.2	0
Hamster	CHO	0	32.0	0
	BHK21	0	386.4	0
Rat	BRL3A	0	1.2	0
Murine	NMuLi	0	12.0	0
	NIH3T3	0	28.8	0
	NIH3T3-hCD81	0	45.5	0

Note. VSVΔG-E1E2, VSVΔG* and VSVΔG*-G were prepared by infecting the CHO cell line expressing both of the chimeric E1 and E2 proteins, normal CHO cells and those transiently expressing VSV-G protein with VSVΔG*-G, respectively. To adjust the infectious titer, VSVΔG*-G was diluted 10^{-3} with DMEM containing 10% FBS. Various cell lines were infected with the pseudotype VSVs from the same batch. The data are representative results from experiments repeated with three batches of viruses.

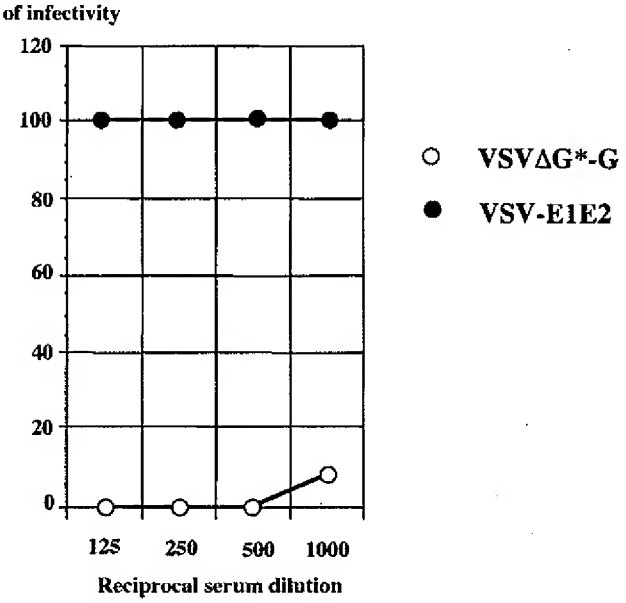

FIG. 5. Neutralization of pseudotype VSVs. A polyclonal antibody against VSV was serially diluted and incubated with 2.5×10^3 IU of VSV-E1E2 or VSVΔG*-G for 30 min at 37°C. The amount of infectious virus remaining was quantified by determining the number of GFP expressing cells.

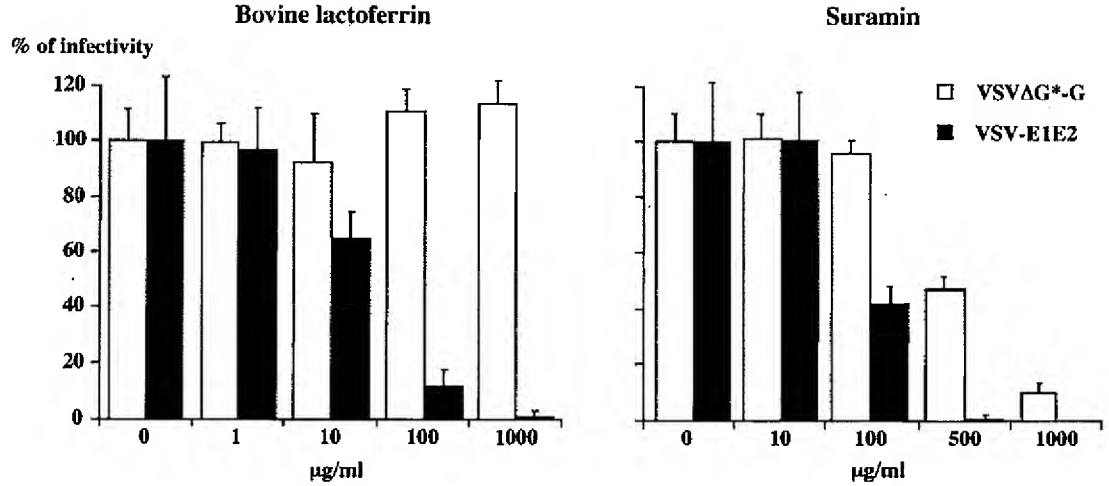

FIG. 6. Effect of bovine lactoferrin and Suramin on the infectivity of pseudotype VSVs. VSV-E1E2 or VSVΔG*-G were pretreated with various concentrations of bovine lactoferrin or Suramin at 37°C for 60 min and then added to HepG2 cells. After 16 h of incubation, the number of the GFP positive cells was determined. The results shown are from three independent assays, with the error bars representing SD.

gested to be the binding receptor for the E2 protein of HCV (Pileri *et al.*, 1998), a NIH3T3 mouse cell line constitutively expressing hCD81 did not exhibit a higher susceptibility of infection for the pseudotyped virus than the parental NIH3T3 cell line. In addition, we recently established an HCV-specific cell fusion assay based on the activation of a reporter gene (Takikawa *et al.*, 2000). The fusion assay utilizes the same CHO cell lines expressing the chimeric HCV envelope proteins as was used in this study. We found that expression of hCD81 in the same NIH3T3 cell line did not elicit cell–cell fusion (Takikawa *et al.*, 2000), even though the truncated E2 protein from the type 1b HCV clone used in this study efficiently bound to the NIH3T3 cell line expressing hCD81 (data not shown). Although hCD81 is expressed on the surface of many human cell lines (Levy *et al.*, 1998), expression levels of hCD81 on the HepG2 cell line are much lower than that of other human cell lines examined (Flint *et al.*, 1999). Nonetheless, HepG2 cells showed the highest susceptibility to the pseudotyped VSV as well as the most cell–cell fusion (Takikawa *et al.*, 2000). These results suggest that expression of hCD81 alone is not sufficient for infection of HCV and that another molecule or cofactor might be required. In the case of HepG2 cells, HCV infection may occur in an hCD81-independent manner.

We found that incorporation of both of the chimeric E1 and E2 proteins into virions was essential for maximal infectivity of the pseudotypes. This finding is different from the data reported by Ray and colleagues in which pseudotyped VSVs possessing either a chimeric E1-G or a chimeric E2-G envelope protein individually was sufficient for virus infectivity on various mammalian cells (Lagging *et al.*, 1998; Meyer *et al.*, 2000). The results by Ray *et al.* are also inconsistent with our previous studies in which expression of the both of the chimeric HCV envelope proteins was essential for maximal cell–cell fusion activity (Takikawa *et al.*, 2000). Furthermore, our E1E2 pseudotyped virus showed no infectivity on BHK cells, whereas the pseudotype viruses generated by Ray's group exhibited the highest infectivity on BHK cells. Although we do not know the reasons for these contradictory results, the differences might be attributable to the system used to produce the pseudotype VSV. We employed a system in which foreign proteins expressed in trans were incorporated into a recombinant VSV that lacked the glycoprotein gene. This system was used previously for the production of infectious pseudotype VSVs possessing the Ebola virus glycoprotein (Takada *et al.*, 1997), measles virus glycoproteins (Tatsuo *et al.*, 2000), and human T-cell leukemia virus type 1 envelope glycoprotein (Okuma *et al.*, 2001). On the other hand, Ray and colleagues used a temperature-sensitive VSV mutant (ts045) for production of the pseudotype VSVs in combination with a recombinant vaccinia virus expressing T7 RNA polymerase. Alternatively, the differences may be due to the type of HCV envelope protein used or the way that the chimeric proteins were constructed.

The envelope proteins of HCV contain signals that retain these proteins in the ER (Cocquerel *et al.*, 1998, 1999). The maturation and budding site of HCV is also believed to be the ER. In this study, we used stably transformed CHO cell lines expressing chimeric HCV envelope proteins instead of a transient expression system to produce the pseudotype VSVs. The size of the E1 protein expressed on the cell surface of the CHO cells was larger (60 kDa) than those expressed in the ER, as reported previously (30–35 kDa) (Fournillier-Jacob *et al.*, 1996; Meunier *et al.*, 1999), and the glycans found on the E1 chimera were processed to complex, endo H resistant forms. Although the exact molecular characteristics of HCV envelope proteins in the virion are not known yet,

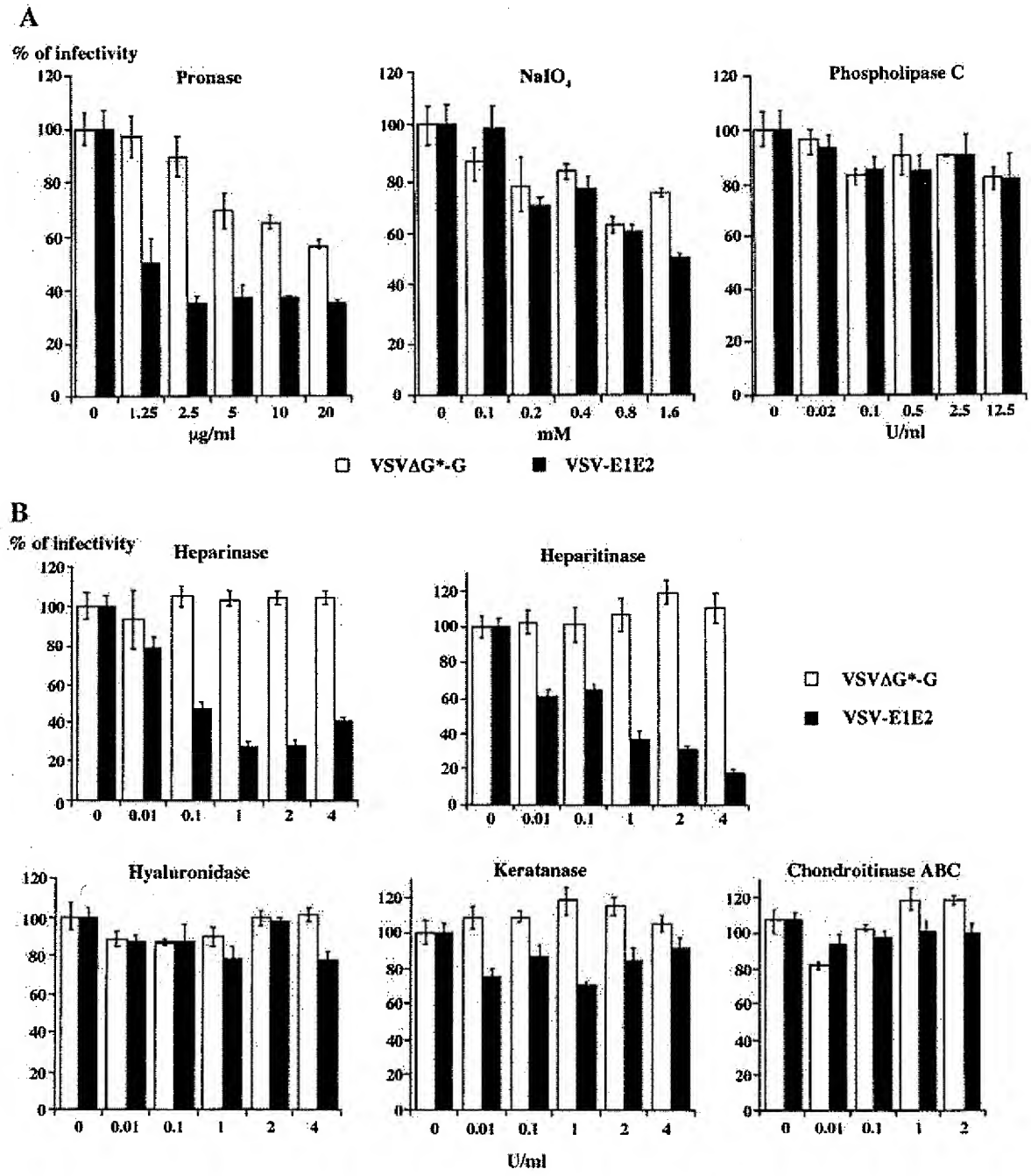

FIG. 7. Effect of chemical modification of HepG2 cells on infectivity of pseudotype VSVs. (A) HepG2 cells were treated with various concentrations of Pronase, sodium periodate, or phospholipase C. (B) HepG2 cells were treated with heparinase, heparitinase, hyaluronidase, keratanase, or chondroitinase ABC. The treated cells were infected with 2.5×10^3 IU of the VSV-E1E2 or VSVΔG*-G, incubated at 37°C for 16 h and then the number of the GFP positive cells was determined. The results shown are from three independent assays, with the error bars representing SD.

the carbohydrates of the envelope proteins on the virions are probably processed into complex type during transport to the cell surface, even if the virions bud into the ER lumen. When we produced pseudotype VSVs using a transient expression system with 293T cells, the infectious titers were lower than those obtained from the CHO cell lines (data not shown). Whether this difference is due to the amount of E1 and E2 expressed on the cell surface or due to differences in glycosylation is not known, but the possibility that the glycosylation state of E1 might be important for infection is intriguing. Several smaller forms of the chimeric E2 protein were detected in the VSV-E2 particles in comparison to that found in VSV-E1E2, suggesting that the chimeric E2 protein was somewhat unstable and susceptible to proteolysis. Although we could not detect an interaction between the chimeric E1 and E2 proteins, coexpression of the two proteins may contribute to their stability.

The effects of chemical modification of HepG2 cells on the infectivity of the pseudotype virus suggest that protein molecules and GAGs on the cell surface play an important role in the binding and/or entry of the pseudotype VSV particles. These cell surface components presumably serve as cellular receptors or cofactors for the virus. The role of GAGs is to bind a diverse group of growth factors, chemokines, enzymes, and matrix components. Many viruses, including herpesviruses (Lycke *et al.*, 1991; WuDunn and Spea, 1989), human immunodeficiency virus-1 (HIV-1) (Mondor *et al.*, 1998; Patel *et al.*, 1993; Roderiquez *et al.*, 1995), picornaviruses (Jackson *et al.*, 1996), and alphaviruses (Byrnes and Griffin, 1998; Klimstra *et al.*, 1998), utilize GAGs to mediate attachment and infection of target cells. Heparin binding proteins are known to interact with heparin via electrostatic interactions generated between the negatively charged sulfate groups on heparin and the positively charged amino acids within the protein (Cardin and Weintraub, 1989; Flynn and Ryan, 1995; Trybala *et al.*, 1996). In dengue virus, both a highly sulfated heparan sulfate and a polysulphonate pharmaceutical, Suramin, were shown to effectively prevent infection of target cells (Chen *et al.*, 1997). HCV envelope proteins were predicted to possess a heparin-binding motif (Chen *et al.*, 1997) and Suramin was shown to be capable of blocking HCV binding to HepG2 cells by using a PCR assay (Garson *et al.*, 1999). However, in this study the effect of Suramin on the infection of pseudotype VSVs was not specific at high concentrations of this compound. Furthermore, it should be noted that additional receptors besides GAGs are involved in binding and entry of many of these viruses. The situation has been most carefully studied with HSV-1, where binding to heparan sulfate is followed by binding to a more specific protein receptor (Montgomery *et al.*, 1996). Sindbis virus was shown to replicate on GAG-deficient pgsA-745 CHO cells and the presence of other receptors was suggested (Byrnes and Griffin, 1998). Although the nature of these additional receptors is not completely clear, it has previously been found that treating cells with either proteases or phospholipases decreases binding of Sindbis virus (Ubol and Griffin, 1991).

Although chimpanzees immunized with E1 and E2 proteins were protected from viral challenge with the homologous HCV strain (Choo *et al.*, 1994), earlier studies had shown that chimpanzees can be readily reinfected, even with homologous strains of HCV (Farci *et al.*, 1992). Therefore, it is believed that cross neutralization between different genotypes will be difficult to achieve (Lemon and Thomas, 1997). Based on these observations, the serum of a chimpanzee immunized with the E1 and E2 proteins of HCV type 1a may not be able to neutralize the pseudotyped virus produced in this study, which possessed the type 1b envelope proteins. In the case of HIV-1 vaccines, it had been widely accepted that multiple envelope protein immunogens might be needed to span the range of HIV sequence diversity (Wrin *et al.*, 1995). However, LaCasse *et al.* recently demonstrated that epitopes with superior immunogenicity are exposed when HIV-1 begins to fuse with host cell membranes. These authors reported that HIV-1 gp120-gp41-CD4 fusion complexes elicited antibodies in transgenic mice which were capable of neutralizing a broad range of primary isolates of HIV (LaCasse *et al.*, 1999). Extending these findings to HCV, it might be possible that fusion intermediates are the critical targets for HCV neutralizing antibodies, and that these antibodies would be effective for a broad range of HCV genotypes.

It has been demonstrated that human as well as bovine lactoferrin binds HCV envelope proteins and the regions responsible for specific interactions between HCV envelope proteins and lactoferrin have been determined (Yi *et al.*, 1997). Furthermore, Ikeda *et al.* demonstrated that bovine lactoferrin prevented HCV infection in human cultured cells (Ikeda *et al.*, 1988, 2000). In this study, we found that bovine lactoferrin exhibited a specific and dose-dependent inhibition of VSV-E1E2 infection, but had no effect on cell infection by VSVΔG*-G. This specific inhibition of VSV-E1E2 infection by bovine lactoferrin suggests that the infection of HepG2 cells by the VSV-E1E2 pseudotypes is likely to be similar to the infection of cells by native HCV virions.

In summary, we have established a system for the production of VSV pseudotypes possessing modified HCV envelope proteins using a CHO cell line constitutively expressing both of the chimeric HCV envelope proteins. In addition to providing information on the nature of cellular receptors used by HCV, the VSV-E1E2 pseudotypes also offer a unique tool to identify potential antiviral agents specific for HCV, as well as a reagent that could be used to analyze HCV-specific neutralizing antibodies or to develop a new class of HCV vaccines.

MATERIALS AND METHODS

Plasmids

The cDNA for the VSV (Indiana serotype) G protein was excised from pGL-1 (Rose and Bergmann, 1982) and cloned into a mammalian expression plasmid pCAGGS (Niwa *et al.*, 1991) under the CAG promoter. The resulting plasmid was designated as pCAG-VSVG. Construction of expression plasmids encoding chimeric E1 (pCAV340V) and E2 protein (pCAV711V) was described in detail (Takikawa *et al.*, 2000). The chimeric E1 and E2 constructs consisted of the ectodomains of E1 or E2 protein of type 1b HCV cDNA clone (NIH-J1) (Aizaki *et al.*, 1998) and the N-terminal signal sequences, transmembrane, and cytoplasmic domains of VSV G protein, as shown in Fig. 1. The chimeric E1 and E2 cDNA clones were excised by *Eco*RI digestion from the pCAV340V and pCAV711V and cloned into pEF-puro and pEF-neo

(Ohashi *et al.*, 1994), respectively. The resulting expression plasmids were designated as pEF-puro E1 and pEF-neo E2.

Cells

CHO cells were transfected with either or both of the pEF-puro E1 and pEF-neo E2 by electroporation (300 V, 960 μF, Bio-Rad Gene Pulser, Bio-Rad), selected with puromycin (Sigma, St. Louis, MO, 10 μg/ml), and/or geneticin (1 mg/ml; Gibco BRL, Gaithersburg, MD) and stable cell lines were obtained. CHOE1, CHOE2, and CHOE1E2 express the chimeric E1, or E2, or both of the proteins, respectively. Various mammalian cell lines were used to examine their susceptibility to infection by the pseudotype VSVs. These included human cell lines (HepG2, FLC4, Huh7, HeLa, 293T), monkey kidney cell lines (CV-1, COS7, and MA104), as well as cell lines derived from other species, including CPK, MDBK, MDCK, BHK21, CHO, BRL3A (rat liver cell), NMuLi (mouse liver cell), NIH3T3, and NIH3T3hCD81, which expresses hCD81. These cells were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO Laboratories, Grand Island, NY) containing 2 mmol of L-glutamine, penicillin (50 IU/ml), streptomycin (50 μg/ml), and 10% fetal bovine serum (FBS) or in the media recommended by the American Type Culture Collection (ATCC). HepG2 cells were purchased from the Dainippon Pharmaceutical Co., Ltd., Osaka, Japan. Huh7 was a gift from the Japanese Cancer Research Resources Bank-Cell, Tokyo. BRL3A and NMuLi were purchased from the ATCC. NIH3T3hCD81 and NIH3T3 were kindly provided by Dr. S. Abrignani, IRIS, Chiron, Siena Italy.

Characterization of the chimeric HCV proteins in CHO cell lines

Expression of the chimeric HCV proteins was analyzed by flow cytometry. Cells were detached, washed with DMEM containing 2% FBS, and incubated with anti-E1 and anti-E2 monoclonal antibodies raised against partially purified recombinant E1 and E2 proteins expressed by recombinant baculoviruses (Matsuura *et al.*, 1994) for 30 min on ice. After washing, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (TAGO, Burlingame, CA) diluted to 1:100 in phosphate-buffered saline (PBS) for 30 min on ice. After washing with PBS, the cells were analyzed by a FACScan flow cytometer (Becton–Dickinson, San Jose, CA). To examine the processing of the chimeric proteins in the CHO cell lines, cells were labeled with 20 μCi of Tran ^{35}S-label (ICN, Irvine, CA) for 15 min after 2 h of starvation with medium deficient in FBS, methionine, and cysteine. The medium was then replaced with normal growth medium, and the radioactively labeled cells were chased for 0, 2, and 4 h, washed twice with PBS, and dissolved in 400 μl of TNE buffer (10 mM Tris–HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10 μg of aprotinin per ml). Cell lysates (100 μl) were suspended in 900 μl of TNE buffer and incubated with 0.5 μg of the anti-E1 or E2 monoclonal antibody and 20 μl of Protein A Sepharose (Pharmacia, Tokyo) [50% suspension (v/v) in TNE buffer] for 1 h at 4°C with rotating. After centrifugation at 8000 *g* for 10 s at 4°C, the pellets were washed twice with TNE buffer. Immunoprecipitated proteins were eluted from Protein A Sepharose in 50 μl of 0.5% SDS and 50 mM 2-mercaptoethanol by boiling for 2 min. The immunoprecipitates were analyzed by SDS–PAGE. To examine the type of oligosaccharides present on the chimeric E1 envelope protein, the CHOE1E2 cell line was pulse-chase labeled and immunoprecipitated with anti-E1 monoclonal antibody. The immunoprecipitates were digested with Endo H or PNGase F following a protocol provided by the manufacturer and the proteins were analyzed by SDS–PAGE.

Production of pseudotype VSVs possessing chimeric HCV envelope proteins

A recombinant VSVΔG*-G was generated by reverse genetics as described previously (Takada *et al.*, 1997). Briefly, BHK cells were infected with a recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3) and then transfected with a T7 expression plasmid encoding a full-length cDNA clone of a recombinant VSV genome, in which the G protein coding region was replaced with the GFP gene (ΔG*), together with plasmids expressing the VSV nucleocapsid protein (N), phosphoprotein (P), polymerase protein (L), and glycoprotein (G). After removal of the vaccinia virus by filtering, the ΔG* virus was passaged on cells transiently expressing G protein only. The VSV-G complemented virus is denoted VSVΔG*-G. To produce ΔG* particles containing the chimeric HCV envelope proteins, the CHO cell lines were infected with VSVΔG*-G at a multiplicity of infection of 5. The virus was adsorbed for 1 h at 37°C and extensively washed three times with DMEM without FBS. After 16 h of incubation at 37°C, culture supernatants were collected and centrifuged to remove cell debris. Pseudotyped VSVs containing the chimeric E1, E2, or E1E2 proteins were recovered after infection of the CHOE1, CHOE2, CHOE1E2 cell lines with VSVΔG*-G, respectively. In addition, the noncomplemented VSVΔG* control virus was recovered from the parent CHO cell line. The VSVΔG*-G was also amplified in CHO cells transfected with pCAG-VSVG. These viruses were partially purified by centrifugation through 25% sucrose and analyzed by immunoblotting. To determine the infectious titer of the pseudotype VSVs, cells were infected with the pseudotype viruses and incubated at 37°C for 16 h. Infectious units (IU) of virus in different cell lines were determined by counting the number of GFP-expressing cells by fluorescence microscopy.

Neutralization assay

Serially diluted antibodies were incubated with 2.5 × 10^3 IU of the pseudotype VSV-E1E2 or VSVΔG*-G for 30 min at 37°C. The amount of infectious virus remaining infectious titer was determined as described above by counting the number of GFP-expressing cells under fluorescence microscopy.

Effects of chemicals on infectivity of pseudotype VSVs

The pseudotype VSV-E1E2 or VSVΔG*-G (2.5 × 10^3 IU) was preincubated with various concentrations of bovine lactoferrin or Suramin at 37°C for 60 min and inoculated into HepG2 cells (4 × 10^4 cells) prepared in a 96-well plate. After absorption for 60 min at 37°C, the cells were washed with DMEM containing 10% FBS three times and incubated at 37°C for 16 h and IU were determined as described above.

Chemical modification of cells

HepG2 cells (4 × 10^4 cells) prepared in a 96-well plate were washed with serum-free DMEM three times and incubated with 25 μl of the serum-free DMEM containing various concentration of Pronase, sodium periodate, phospholipase C, and glycosaminoglycan lyases at 37°C for 20, 60, 30, and 60 min, respectively. To stop the treatment, an equal volume of DMEM containing 10% FBS was inoculated. After washing with serum-free DMEM once, the cells were infected with 2.5 × 10^3 IU of the pseudotype VSV-E1E2 or VSVΔG*-G and incubated at 37°C for 16 h and the number of the GFP positive cells was determined.

Reagents

Endo-β-*N*-acetylglucosaminidase H (Endo H), peptide-*N*-glycosidase F (PNGase F), and phospholipase C (*Bacillus cereus* Grade I) were obtained from Boehringer Mannheim, Germany. Pronase and sodium periodate ($NaIO_4$) were obtained from Aldrich, Tokyo, Japan. Heparitinase (*Flavobacterium heparinum*), heparinase (*F. heparinum*), hyaluronidase (*Streptomyces hyalurolyticus*), chondroitinase ABC (*Proteus vulgaris*), and keratanase (*Pseudomonas* sp.) were kindly provided by Seikagaku Corp., Tokyo, Japan. Anti-VSV polyclonal antibody was purchased from Lee Biomolecular Research Laboratories, Inc. (San Diego, CA). Bovine lactoferrin and Suramin were obtained from Sigma Chemical Co.

ACKNOWLEDGMENTS

We thank Dr. J. K. Rose for providing the pGL-1 plasmid, Dr. S. Abrignani for the NIH3T3 cell line expressing hCD81, and Dr. M. Houghton for the immunized chimpanzee serum. We also thank T. Mizoguchi and Yuko Matsuura for secretarial work and S. Ogawa for technical assistance. This work was supported by a Research on Advanced Medical Technology grant (Y.M.) and a Research on Emerging and Re-Emerging Infectious Diseases grant (T.M.) from the Ministry of Health and Welfare, Organization for Pharmaceutical Safety and Research grant (OPSR) (T.M.), and Grant-in Aid from the Ministry of Education, Science, Sports, and Culture of Japan (Y.M.), and by NIH Grants GM-53726 and AI-48216 (M.A.W.).

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Virology 302, 294–298 (2002)
doi:10.1006/viro.2002.1625

Mechanism of Neutralization of Influenza Virus Infectivity by Antibodies

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Received April 18, 2002; returned to author for revision May 28, 2002; accepted June 17, 2002

We have determined the mechanism of neutralization of influenza virus infectivity by three antihemagglutinin monoclonal antibodies, the structures of which we have analyzed before as complexes with hemagglutinin. The antibodies differ in their sites of interaction with hemagglutinin and in their abilities to interfere *in vitro* with its two functions of receptor binding and membrane fusion. We demonstrate that despite these differences all three antibodies neutralize infectivity by preventing virus from binding to cells. Neutralization occurs at an average of one antibody bound per four hemagglutinins, a ratio sufficient to prevent the simultaneous receptor binding of hemagglutinins that is necessary to attach virus to cells. © 2002 Elsevier Science (USA)

Key Words: influenza; neutralization; hemagglutinin; structure; antibody.

INTRODUCTION

Protection against influenza is mediated by antihemagglutinin (HA) antibodies which also display virus infectivity neutralization *in vitro*. Two lines of evidence suggest that antibodies that participate in neutralization are an important component of those that lead to protection from infection: $F(ab')_2$ preparations, devoid of the Fc-dependent functions of the IgGs, were found to cure infections in SCID mice (Palladino *et al.*, 1995) and antibodies that do not neutralize have generally been found to be incapable of curing infections in these animals (Gerhard *et al.*, 1997). There are, however, uncertainties about the mechanism of neutralization of influenza virus infectivity (for reviews, see Dimmock, 1995; Klasse and Sattentau, 2001; Parren and Burton, 2001). We have therefore determined the structures of complexes of HA with three antibodies that bind to three distinct epitopes on HA (Barbey-Martin *et al.*, 2002; Bizebard *et al.*, 1995; Daniels *et al.*, 1983, 1987; Fleury *et al.*, 1999; Skehel *et al.*, 1984) (Fig. 1) and studied the mechanism by which they neutralize infectivity.

HA is involved in two steps of the process of influenza virus infection. It binds the virus to its cellular receptors, sialic acid residues of glycolipids, or glycolipids, and, following endocytosis, it mediates the fusion of viral and cellular membranes to permit entry of the genome–transcriptase complex into the cell. HA is a trimer of identical subunits. Structurally, each subunit consists of a membrane-proximal helix-rich stem structure and a membrane-distal receptor-binding globular domain (Wiley and Skehel, 1987). The epitopes recognized by the three antibodies we have studied are located on the receptor-binding domain (Fig. 1). Two of them overlap with the receptor-binding site and block access to it (Barbey-Martin *et al.*, 2002; Bizebard *et al.*, 1995), while the third is distant from the site (Fleury *et al.*, 1999). The three antibodies also differ in their abilities to prevent the structural transition of HA that is required for fusion of virus and cellular membranes: one of them blocks this transition, the other two do not (Barbey-Martin *et al.*, 2002).

These three antibodies, therefore, are representative of the range of neutralizing antibodies that react with hemagglutinin and have provided the opportunity for us to study the relationship of neutralization to the inhibition of two successive steps in viral entry into the cell, in a structurally defined context. We show that there is a direct correlation between neutralization of virus infectivity and inhibition of virus binding to cells and determine for each of the antibodies the number of molecules that is required to achieve neutralization.

RESULTS AND DISCUSSION

The number of antibodies bound to virus in neutralization

The number of antibody molecules bound to virus was measured by incubating ^{125}I-labeled antibody with virus and separating bound from unbound antibody by centrifugation. The antibody concentrations chosen covered the range of concentrations in which neutralization of infectivity varies between 0 and 100% and the concen-

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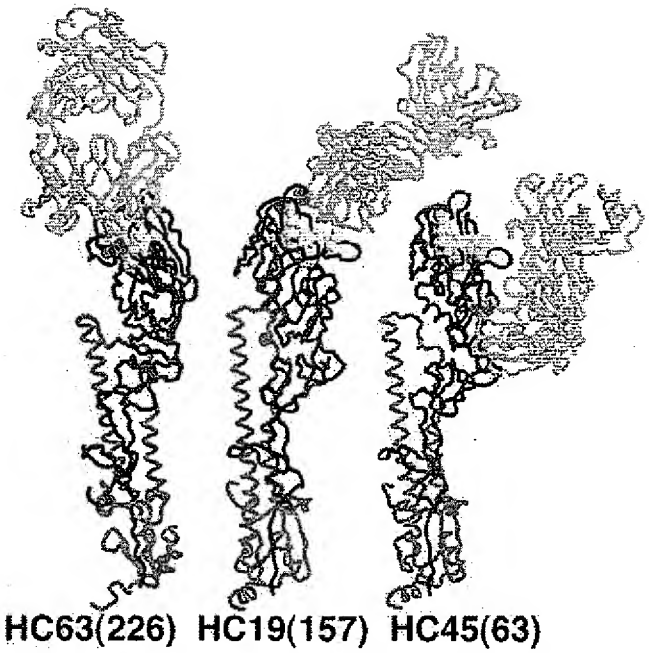

FIG. 1. The Fab–HA complexes in this study. Ribbon diagrams of the complexes showing one X31 HA monomer (each monomer contains two polypeptide chains; one in blue forming the receptor-binding domain and the other in red forming the stem domain) and, from left to right, the HC63(226), HC19(157), and HC45(63) Fabs (in green). Amino acids in the receptor-binding site are shown as yellow space-filling models. Each antibody is designated by a number as in previous work together with (in parentheses) the residue in the sequence in which a mutation has been identified that allows a variant virus to escape from neutralization of infectivity by the antibody. These residues are colored in red in the complexes.

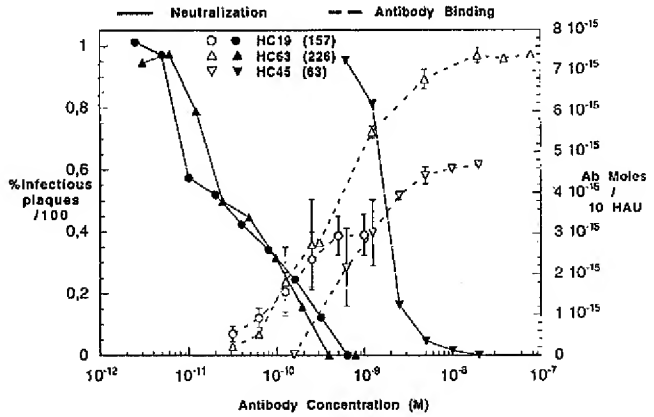

FIG. 2. The relation between the number of antibody molecules bound to a virus particle and neutralization. The ratios of the number of virus plaques to the number of plaques formed without antibody (plain lines, filled symbols) and the number of antibody molecules bound to 10 HAU of X31 virus (dashed lines, open symbols) are plotted on a semilogarithmic scale as a function of antibody concentration (HC19(157): ○; HC63(226): △; HC45(63): ▽). Each point of the latter curves is the average of three independent experiments; plaque number ratios are the average of two experiments.

tration required for each antibody to saturate the virus. The data in Fig. 2 indicate that iodination of the antibodies does not significantly affect virus–antibody interactions (compare Fig. 3). We also checked that antibody does not significantly detach from virus during separation of the complex by comparing our results to those obtained by centrifugation through a sucrose solution in which ^{125}I-labeled antibody was present at the same concentration as that incubated with virus.

Two of the antibodies, HC19(157) and HC45(63), neutralize viral infectivity at a concentration at which they saturate the virus (5×10^{-10} M and 10^{-8} M, respectively), whereas the third antibody, HC63(226), neutralizes at an antibody concentration of 4×10^{-10} M, lower than the concentration required for saturation (2×10^{-8} M). These results are consistent with observations made on the basis of the structures, that whereas bound HC63(226) Fabs extend from the hemagglutinin within the space projected radially from a trimer, HC19(157) and HC45(63) both bind on the sides of the trimer so that their complexes occupy more space on the virus surface than the trimer (Fig. 1). As a consequence of the limited space available on the virus surface, saturation occurs at an antibody:HA ratio that depends on the geometry of the specific HA–antibody complex. Obviously, saturation of virus by all antibodies with the ability to neutralize infectivity occurs at a concentration higher than or equal to that required for neutralization, which is what we find.

The antibody:HA spike ratio at which complete neutralization by HC45(63) is achieved is ca. 1:3 ± 1. For HC19(157) and HC63(226), this ratio is ca. 1:5 ± 1.5. Neutralization by HC45(63) is therefore less efficient than by HC19(157) or HC63(226) for two reasons: HC45(63) has a lower avidity for hemagglutinin on the virus surface

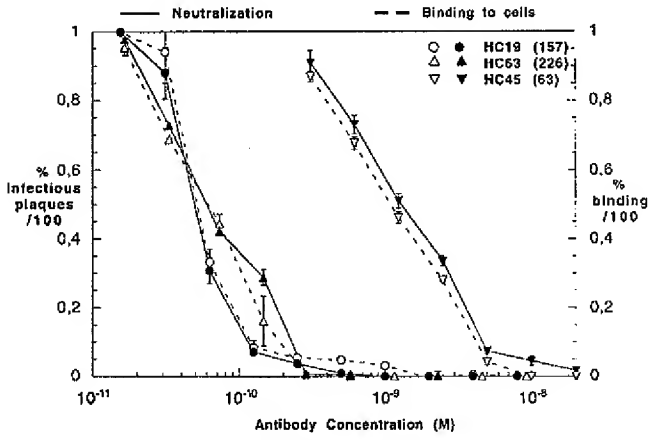

FIG. 3. The relation between inhibition of virus binding to cells by antibodies and neutralization. The ratios of the number of virus plaques to the number of plaques without antibody (plain lines, filled symbols) and the ratio of cell-bound virus to cell-bound virus without antibody (dashed lines, open symbols) are plotted on a semilogarithmic scale as a function of antibody concentration (HC19(157): ○; HC63(226): △; HC45(63): ▽). Each point is the average of three independent experiments.

(Fleury *et al.*, 1999), and more HC45(63) antibody molecules than HC19(157) or HC63(226) antibodies are required to bind to a virus to neutralize its infectivity.

Antibodies block virus attachment to cells

Viral attachment to cells is the first step in the infectious cycle and its inhibition would appear to be an effective way of preventing infection. Despite this, inhibition of virus attachment to cells by antibodies has been reported only rarely as the major contributor to infectivity neutralization (see Ugolini *et al.*, 1997 for an example and Dimmock, 1995 for a review). There are, however, several examples of inhibition of virus attachment by neutralizing antibodies (Colonno *et al.*, 1989; Flamand *et al.*, 1993; He *et al.*, 1995; Smith *et al.*, 1993). Using mixtures of antibodies and labeled virus, we determined the amount of radioactive virus that bound to cells as a function of antibody concentration. In parallel we determined the reduction in the number of infectious particles caused by mixing a constant amount of virus with antibodies at different concentrations. The data presented in Fig. 3 demonstrate a direct correlation between inhibition of virus binding to cells and neutralization of infectivity. From our structural analyses two of the antibodies, HC19(157) and HC63(226), bind to the receptor-binding site. Since their affinities for hemagglutinin (K_D of Fabs 5×10^{-10} M (Fleury *et al.*, 1999) and 1.7×10^{-10} M, respectively) are much stronger than the affinities of the receptor-binding site for sialyllactose receptor analogues (K_D 2×10^{-3} M) (Sauter *et al.*, 1989), these antibodies effectively block receptor binding. The third antibody HC45(63) also has a strong affinity for HA (K_D of Fab: 10^{-9} M) (Fleury *et al.*, 1999) but binds to HA at a distance from the receptor-binding site; the distance of the nearest Fab atom to the receptor-binding site is 17 Å (Fleury *et al.*, 1999). Nevertheless, its inhibition of virus binding at different concentrations correlates with its neutralization of infectivity. Because of the low affinity of the HA receptor-binding site for the virus receptor, several HAs bind to a receptor upon attachment of a virus particle to a cell. Presumably in the case of HC45(63), the bound immunoglobulin, because of its large size, prevents this simultaneous binding. Since HC45(63) binds outside the receptor-binding site and closer to the virus membrane than antibodies HC19(157) and HC63(226), one would predict that it inhibits simultaneous binding of several HAs to a viral receptor less efficiently than these antibodies. Indeed, less HC19(157) or HC63(226) antibodies than HC45(63) antibodies are required to bind to a virus particle to neutralize its infectivity (see above).

One of the three antibodies we studied, HC63(226), cross-links *in vitro* monomers in the hemagglutinin trimer (Barbey-Martin *et al.*, 2002) and prevents the low pH-activated structural transition required for membrane fusion (Skehel and Wiley, 2000). The epitopes of this antibody and of HC19(157) overlap with the receptor-binding site and both antibodies have similar affinities for HA but, as opposed to HC63(226), HC19(157) does not interfere with the low pH-activated structural change. Since fusion in endosomes follows receptor-binding and endocytosis, in order for fusion inhibition to contribute to neutralization, it would have to occur at an antibody concentration at which virus still binds to cells. In the case of antibody HC63(226) this would be at a very low antibody concentration since complete neutralization by HC63(226) and inhibition of virus binding to cells is achieved at an antibody concentration of 5×10^{-10} M. At this concentration the ratio of the number of antibodies bound to virus to the number of hemagglutinin spikes per virus is close to 1:5 (see above and Fig. 2). In these conditions the maximum proportion of hemagglutinin trimers that are internally cross-linked is also 1:5, so that about 80% of the hemagglutinins could still undergo the low pH structural change. It is nevertheless possible that if the HAs in virus particles are fixed in the plane of the membrane and if the mechanism of membrane fusion requires the cooperation of a number of HAs, inhibition of fusion could contribute to infectivity neutralization. However, the direct correlation between neutralization of infectivity and inhibition of binding to cells by the antibodies studied here (Fig. 3) suggests that they neutralize infectivity by preventing receptor binding and that inhibition of membrane fusion does not contribute significantly to neutralization.

Concluding remarks

Our results highlight two features of the antibody inhibition of virus binding to cells which affect neutralization of infectivity:

First, the average number of virus-bound antibodies required for neutralization is between 60 and 110 with an estimated number of HA trimers per virion of about 300 (Cusack *et al.*, 1985) (larger estimates have also been proposed by Taylor *et al.*, 1987). This is not inconsistent with the single-hit kinetics of viral neutralization that have been observed (Schofield *et al.*, 1997), as noted and reviewed elsewhere (Dimmock, 1995; Klasse and Moore, 1996; Parren and Burton, 2001).

Second, the antibody concentration required to achieve neutralization, between 2×10^{-10} M for HC19(157) and 10^{-8} M for HC45(63), is significantly higher than the avidities of the antibodies for viral HA (HC19(157): $K_D = 6 \times 10^{-12}$ M; HC45(63): $K_D = 100 \times 10^{-12}$ M) (Fleury *et al.*, 1999). These were measured at low virus occupancy; as more antibody molecules are bound to virus and crowding on the viral surface increases, their avidity for virus HA is expected to decrease. This would explain the observed difference between antibody avidity for viral HA and the antibody concentration required for neutralization of viral infectiv-

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Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 9339–9343, October 1992
Medical Sciences

Recombinant human Fab fragments neutralize human type 1 immunodeficiency virus *in vitro*

(antibody repertoire/passive immunization/virus neutralization/phage surface expression)

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Communicated by Frank J. Dixon, June 26, 1992

ABSTRACT **A panel of 20 recombinant Fab fragments reactive with the surface glycoprotein gp120 of human type 1 immunodeficiency virus (HIV-1) were examined for their ability to neutralize MN and IIIB strains of the virus. Neutralization was determined as the ability of the Fab fragments to inhibit infection as measured in both a p24 ELISA and a syncytium-formation assay. One group of closely sequence-related Fab fragments was found to neutralize virus in both assays with a 50% neutralization titer at ≈1 μg/ml. Another Fab neutralized in the p24 ELISA but not in the syncytium assay. The other Fab fragments showed weak or no neutralizing ability. The results imply that virion aggregation or crosslinking of gp120 molecules on the virion surface is not an absolute requirement for HIV-1 neutralization. Further, all of the Fab fragments were shown to be competitive with soluble CD4 for binding to gp120 and yet few neutralized the virus effectively, implying that the mechanism of neutralization in this case may not involve receptor blocking. The observation of a preponderance of high-affinity Fab fragments with poor or no neutralizing ability could have implications for vaccine strategies.**

The binding of antibodies to viruses can result in loss of infectivity or neutralization and, although antibodies are not the only defense mechanism against viruses, it is widely accepted that antibodies have an important role to play (reviewed in ref. 1). However, understanding of the molecular principles underlying antibody neutralization is limited and lags behind that of the other effector functions of antibody (2). Such understanding is required for the rational design of vaccines and for the most effective use of passive immunization for prophylaxis or therapy. This is particularly urgent for the human immunodeficiency viruses (HIVs).

A number of studies have led to the general conclusion that viruses are neutralized by more than one mechanism and the one employed will depend on factors such as the nature of the virus, the epitope recognized, the isotype of the antibody, the cell receptor used for viral entry, and the virus/antibody ratio (1). The principal mechanisms of neutralization can be considered as (*i*) aggregation of virions, (*ii*) inhibition of attachment of virus to cell receptor, and (*iii*) inhibition of events following attachment, such as fusion of viral and cellular membranes and secondary uncoating of the virion. One of the important features of the third mechanism is that it may require far less than the approximately stoichiometric amounts of antibody expected for the first two mechanisms, since occupation of a small number of critical sites on the virion may be sufficient for neutralization. For instance, it has been shown that neutralization of the influenza A virion obeys single-hit kinetics (3).

Intensive studies have been carried out on antibody neutralization of HIV-1 (reviewed in ref. 4). Most have focused on a single linear epitope in the third hypervariable domain of the viral envelope glycoprotein gp120 known as the V3 loop. Antibodies to this loop are suggested to neutralize by inhibiting fusion of viral and cell membranes. Binding to the loop resulting in neutralization can occur before virus–cell interaction (M. Merges and P. L. N., unpublished work) or after binding of gp120 to CD4 (5–8). Features of the V3 loop are sequence variability within the loop (9–11) and sensitivity of neutralizing antibodies against the loop to sequence variations outside the loop (4, 11–13). Hence anti-V3 loop antibodies are often strain-specific and mutations in the loop *in vivo* may provide a mechanism for viral escape from antibody neutralization. Recently considerable interest has focused on antibodies capable of blocking CD4 binding to gp120—these are loosely described as "antibodies to the CD4 binding region." A number of groups have described these antibodies as (*a*) reacting with conformational (i.e., nonlinear) epitopes, (*b*) reacting with a wide range of virus isolates, and (*c*) being the predominant neutralizing antibodies in humans after longer periods of infection (14–19). Neutralizing antibodies of this type would appear to present a promising target for potential therapeutics. The mechanism(s) of neutralization of these antibodies is unknown, although there is some indication that they may not act by blocking the attachment of virus, since a number of mouse monoclonal antibodies inhibiting CD4 binding to gp120 are either nonneutralizing (20) or only weakly neutralizing (21).

The generation of human monoclonal antibodies against the envelope of HIV-1 (22) through the use of combinatorial libraries allows another approach to the problem of neutralization. Given the lack of a three-dimensional structure for gp120 and the complexity of the virus, the approach seeks to explore neutralization at the molecular level through the behavior of related antibodies. This is possible because (*i*) the combinatorial approach allows the rapid generation of large numbers of human antibodies, (*ii*) the antibodies (Fab fragments) are expressed in *Escherichia coli* and can readily be sequenced, and (*iii*) antibodies have similar sequences and common structural motifs, allowing functional differences to be meaningfully correlated with primary structure.

Here we describe neutralization studies on 20 recombinant human Fab fragments against gp120, all of which are strain-crossreactive and inhibited by CD4 from binding to gp120.

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Abbreviations: HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cell.

We show that neutralization is not effected by virus aggregation or crosslinking of gp120 molecules on the virion surface and is not correlated with blocking of the interaction between soluble CD4 and recombinant gp120.

MATERIALS AND METHODS

Generation of Soluble Fab Fragments Reactive with gp120. The preparation of RNA from the bone marrow lymphocytes of an HIV-1-seropositive individual, construction of an IgG1(κ) Fab library on the surface of phage by use of the pComb3 system, panning of the library against recombinant gp120 from the IIIB strain of HIV-1, and selection of phagemids expressing soluble Fab fragments that react with gp120 in ELISA assays are described in ref. 22, and the methodology is described in refs 22–24.

Competition ELISAs. Relative Fab binding affinities were estimated by competition, for Fab fragments, of free gp120 with gp120 coated on ELISA wells (22, 25). The ability of soluble CD4 (American Biotechnologies, Columbia, MD) to compete with Fab fragments for gp120 coated on ELISA wells was carried out using similar procedures.

Estimation of Fab Concentrations in Supernatants. This was carried out by sandwich ELISA (26).

Purification of Fabs. One-liter cultures of *E. coli* in super broth (23) containing carbenicillin (50 μg/ml) and $MgCl_2$ (20 mM) were inoculated with appropriate clones, induced 7 hr later with 2 mM isopropyl β-D-thiogalactopyranoside, and grown overnight at 30°C. The cell pellets were sonicated and the supernatant was concentrated to 50 ml. The filtered supernatants were loaded on a 25-ml protein G–anti-Fab column, washed with 120 ml of buffer at 3 ml/min, and eluted with citric acid at pH 2.3. The neutralized fractions were then concentrated, exchanged into 50 mM Mes (pH 6.0), and loaded onto a 2-ml Mono S column (Pharmacia) at 1 ml/min. A gradient of 0–500 mM NaCl was run at 1 ml/min, and the Fab fragments were eluted in the range of 200–250 mM NaCl. After concentration, the Fab fragments were positive when titered by ELISA against gp120 and gave a single band at 50 kDa by SDS/10–15% PAGE. Concentration was determined from absorbance measurement at 280 nm by using an extinction coefficient (1 mg/ml) of 1.4.

Neutralization Assays. *p24 ELISA.* Diluted tissue culture supernatants of HIV-1 IIIB- or MN-infected peripheral blood mononuclear cells (PBMCs) [50 $TCID_{50}$ (50% tissue culture infectious dose) per 100 μl] were incubated for 2 hr at 37°C with serial (1:2) dilutions, beginning at a dilution of 1:20, of recombinant Fab supernatants or controls. The latter included human neutralizing sera, a known human neutralizing monoclonal antibody [2F5 (27)] and the Fab fragment derived from that antibody by papain digestion, and a known mouse neutralizing monoclonal antibody and its $F(ab')_2$ fragment (28, 29). PBMCs (10^5) were added to the virus/antibody mixture and incubated for 1 hr at 37°C. Thereafter the cells were washed and incubated in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum, 1.5 mM glutamine, antibiotics, and interleukin 2. Medium was changed at days 1 and 4. Seven days postinfection, supernatants were collected and analyzed by HIV-1 p24 antigen-capture ELISA (30). Neutralization was defined as positive if an 80% or greater reduction of optical density at 490 nm in the culture supernatant occurred as compared to negative Fab or negative human serum. Tests with all Fab fragments, monoclonal antibodies, and sera were repeated on at least two occasions.

Quantitative infectivity assay based on syncytium formation. A quantitative neutralization assay with the MN strain of HIV-1 was performed as described (31). Monolayers of CEM-SS target cells were cultured with virus, in the presence or absence of antibody, and the number of syncytium-forming units was determined 3–5 days later. An equivalent amount of virus was used in the assays to allow direct comparison of the various antibody concentrations tested. The assays were repeatable over a virus-surviving fraction range of 1 to 0.001 within a 2- to 4-fold difference in the concentration of antibody ($P < 0.001$).

Nucleic Acid Sequencing of Variable Domains of Fab Fragments. Nucleic acid sequencing was carried out on double-stranded DNA by using Sequenase 1.0 (United States Biochemical) and the appropriate primers hybridizing to sequences in the $C_\gamma 1$ (SEQGb, 5′-GTCGTTGACCAGGCAGCCCAG-3′) or C_κ (SEQKb, 5′-ATAGAAGTTGTTCAGCAGGCA-3′) constant domain. Alternatively, sequencing employed single-stranded DNA and the T3 primer (5′-ATTAACCCTCACTAAAG-3′; ref. 32) or one hybridizing to a sequence in the leader sequence (KEF, 5′-GAATTCTAAACTAGCTAGTTCG-3′).

RESULTS AND DISCUSSION

Twenty clones were selected from the Fab library for their ability to bind to glycosylated recombinant gp120 from the IIIB strain of HIV-1. Fab supernatants were prepared and the approximate concentrations of Fab were determined by ELISA as described. Since the Fab fragments are expressed in *E. coli* and the fraction of correctly assembled protein can vary, the amount of Fab reacting with gp120 was also assessed by ELISA titration. Supernatants were then tested for neutralizing ability in infectivity assays employing MN and IIIB strains of HIV-1 and using either p24 levels or syncytium formation as a readout of infection. Assays were

Table 1. Neutralization of HIV-1 by recombinant Fab fragments

Antibody	Fab, μg/ml	Reciprocal of ELISA titer	p24 assay		Syncytium assay
			MN	IIIB	(MN)
Clones					
1	1.8	8	–	–	–
2	3.1	64	–	–	–
3	4.1	32	–	–	–
4	25.0	16	40	80	>128
5	2.4	128	–	–	–
6	4.0	64	–	–	–
7	4.3	64	20	20	>32
8	14.0	256	20	20	–
11	11.0	128	–	–	–
12	6.0	64	80	40	>128
13	6.1	128	80	80	–
18	0.9	128	–	20	–
20	6.9	256	–	–	32
21	8.5	32	20	20	32
22	8.6	64	20	20	–
24	0.7	32	–	–	–
27	10.0	64	20	20	32
29	16.0	1024	–	–	–
31	9.3	128	–	–	–
35	8.9	64	–	–	–
2F5 mAb	10.0		40	160	
2F5 Fab	5.0		40	20	
F58 mAb	10.0		160	40	
F58 $F(ab')_2$	200.0		40	20	

In this experiment aliquots of the same supernatant preparations were used in p24 and syncytium assays with HIV-1 IIIB- and MN-infected cells. Values for these assays indicate neutralization titers. For the p24 assay the titer corresponds to the greatest dilution producing >80% reduction in absorbance in ELISA. For the syncytium assay, Fab fragments 4 and 12 produced >95% neutralization at a 1:4 dilution of supernatant and 80% and 70% reduction at a 1:128 dilution, respectively. –, No neutralization at 1:20 dilution in the p24 assay and 1:16 in the syncytium assay (with most clones showing no detectable neutralization at a 1:4 dilution).

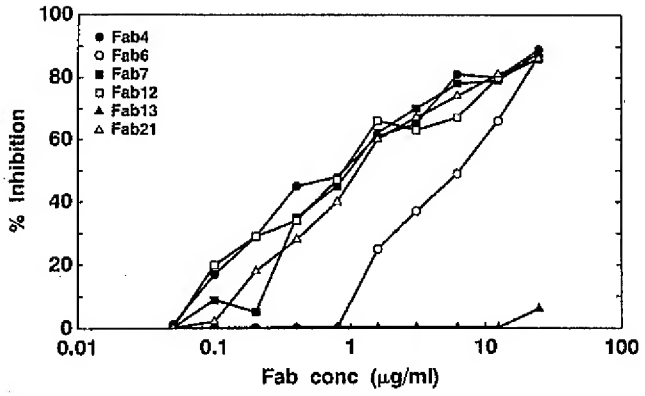

FIG. 1. Neutralization of HIV-1 by purified Fab fragments. Results shown are derived from the syncytium assay using the MN strain. The p24 assay indicated >80% neutralization of HIV-1 MN strain for Fab 4 at 3 μg/ml, Fab 7 at 15 μg/ml, Fab 12 at 3 μg/ml, Fab 13 at 4 μg/ml, and Fab 21 at 7 μg/ml. For the IIIB strain, >80% neutralization was observed for Fab 4 at 13 μg/ml, Fab 7 at 15 μg/ml, Fab 12 at 7 μg/ml, and Fab 21 at 14 μg/ml.

generally repeated at least twice with reproducible results. For the data reported in Table 1, the Fab supernatants were divided into two parts, one being used in the p24 assay and the other in the syncytium assay. Table 1 shows that two Fab fragments, 4 and 12, are effective neutralizers in both types of assay. These Fab fragments have also been shown to neutralize infection by IIIB and RF strains in a PCR-based assay of proviral integration (L. Montaner, S. Gordon, C. F. B., and D. R. B., unpublished work). Fab 13 is consistently effective in the p24 assay but not in the syncytium assay. A number of other clones show lower levels of neutralizing ability.

Fab fragments were purified from a selection of some of the clones and used in both neutralization assays. As shown in Fig. 1, Fab fragments 4 and 12 are again effective in both assays at similar levels, with, for example, 50% inhibition of syncytium formation at an Fab concentration of ≈20 nM (1 μg/ml). Fab fragments 7 and 21 are equally as effective in the syncytium assay but somewhat less so in the p24 assay. Fab 13 neutralizes in the p24 assay at 4 μg/ml but is ineffective in the syncytium assay at 25 μg/ml.

A number of conclusions arise from the data of Table 1 and Fig. 1. It is apparent that HIV-1 can be neutralized without virion aggregation or crosslinking of gp120 molecules on the virion surface, since monovalent Fab fragments are effective. To further confirm this finding we generated an Fab fragment by papain digestion of a known neutralizing human monoclonal antibody. As shown in Table 1, the Fab fragment was approximately equally effective as the whole IgG in neutralization of the MN strain of HIV-1. This is consistent with results on Fab fragments prepared from two mouse monoclonal antibodies to the V3 loop (M. Merges and P. L. N., unpublished work). An $F(ab')_2$ fragment of a mouse monoclonal antibody was somewhat less effective than the parent IgG in neutralization of the MN strain. Interestingly the fragments from these control antibodies were relatively poor in neutralizing the IIIB strain of HIV-1. Table 1 and Fig. 1 also show that there appears to be a difference between the two assays employed, since Fab 13 is consistently effective in one assay but not the other. The principal variables are the incubation time of the virus and antibody prior to infection (2 hr for the p24 assay and 0.5 hr for the syncytium assay), the amount of virus used for infection, the cells used to propagate virus (human PBMCs for the former and H9 cells for the latter), and the cells infected (human PBMCs for the former and CEM.SS cells for the latter). Of these, there is a strong possibility that the MN virus used in the two assays, having been passaged through different cells, is critically different.

To explore the relationship between neutralizing and weakly or nonneutralizing Fabs, the variable domains of the 20 clones were sequenced. A detailed comparison of the sequences will be provided elsewhere (C.F.B., T. A. Collet, P. Roben, J. Binley, D. Hoekstra, D.C., T. M. Jones, R. A. Williamson, N. L. Haigwood, A. Satterthwait, I. Sanz, and D.R.B., unpublished work). The heavy-chain sequences can be organized into six groups where each member of a group has an identical or very similar third complementarity-determining region, with a limited number of differences elsewhere. When the light chains are constrained into the groupings defined by their heavy-chain partners, considerable light-chain sequence variation is observed. This phenomenon of chain promiscuity has been observed previously (22, 33–36) and can be appreciated by reference to Fig. 2. Marked neutralizing ability is confined to

Group 1

Heavy Chain

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
hiv4	LEQSGAEVKKPGASVKVSCQASGYRFS	NFVIH	WVRQAPGQRFEWMG	WINPYNGNKEFSAKFQD	RVTFTADTSANTAYMELRSLRSADTAVYYCAR	VGPYSWDDSPQDNYYMDV	WGKGTTVIVSS
hiv12	.....	.....	.....	.....	.....	.....	.....
hiv7	.....	.....	.....	.....	.....D.....I.....	....T.....	.....K.....
hiv21	.....	.....	.....	.....	.....A.....T...I.....	....T.....	.....K.....

Light Chain

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
hiv4	MAELTQSPGTLSLSPGERATFSC	RSSHSIRSRRVA	WYQHKPGQAPRLVIH	GVSNRAS	GISDRFSGSGSGTDFTLTITRVEPEDFALYYC	QVYGASSYT	FGQGTKLEIKR
hiv12	.....A.....	.....R	.....	.....	.....	.....	.....
hiv7	.....T.....L..	.T.....L.	...V.G.....L.Y	.....G	..P.....S.L.....V...	.Q..S.R..	.....INE
hiv21	.....A.....D.....	....N.....	.....	.....	.....L.....	.....	.....DF.

Group 2

Heavy Chain

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
hiv13	LEQSGEAVVQPGTSLRLSCAASGFTFR	NYAMH	WVRQAPGKGLEWVA	LIKYDGRNKYYADSVKG	RFSISRDNSKNTLYLEMNSLRAEDTAVYYCAR	DIGLKGEHYDILTAYGPDY	WGQGALVTVSS
hiv8	..E.....R.....I..	.....	.....	.....	..T.....Q.....	.....H..	....T...
hiv18	..E.....R.....I..	.....	.....	.....	..T.....Q.....	.....	....T.....
hiv22	..E.....	.....	.....	.....	.....	.....	.....
hiv27	..E.....R.....I..	.....	.....	...S.....	..T.....Q.....	.....	....T.....

Light Chain

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
hiv8	MAELTQSPSSLSASVGDRVTITC	RASQSISNYLN	WYQQKPGKAPKLLIY	AASSLQR	GVPSRFSGSGSGTDFTLSISSLQPEDFATYYC	QQSYSIPPLT	FGGG
hiv22	.....	.....S...	.....	.....S	.....T.....	.....T.YT	..Q.TKLEIK
hiv27	.....	.....S...	.....	.....S	.....T.....	.....T.QT	..Q.TKLEIKR
hiv18	.....	....N.N....	.....E.....H	T.FN..S	.....TA...E...T.R.....	.....T.YT	..Q.TKLEIND
hiv13	.....	Q...D.R....	.....	D..NSET	.....R...FT.....V.....	..HQNV.LT	....TKVEIKR

FIG. 2. Amino acid sequence comparison of the variable domains of two groups of human anti-gp120 Fabs. Groupings are made on the basis of similarities in heavy-chain sequences. Dots indicate identity with the first sequence in each section. FR, framework region; CDR, complementarity-determining region.

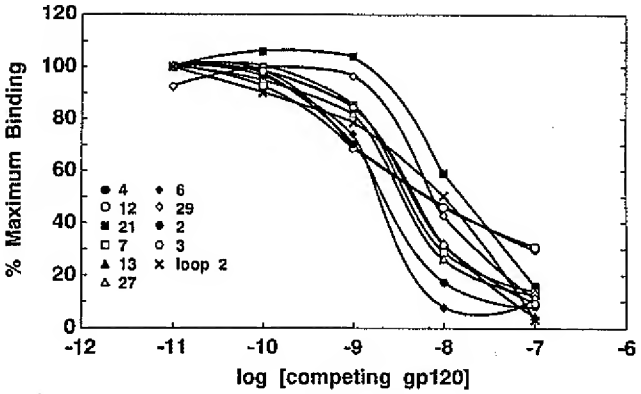

FIG. 3. Relative affinities of Fab fragments for gp120 (IIIB) as illustrated by inhibition ELISA. Fab fragments 27, 6, 29, 2, and 3 are all prototype members of the different groups discussed in the text. Loop 2 is an Fab fragment that was selected from the same library as the other Fab fragments that recognizes the V3 loop. In this case competition was carried out with gp120 from the SF2 strain.

two groups of sequences. The first group consists of Fab fragments 4, 7, 12, and 21, which have very similar heavy and light chains. The second group consists of Fab fragments 8, 13, 18, 22 and 27. Only Fab 13 shows marked neutralizing ability, although the others show some weaker activity. Interestingly, in this group Fab 13 does have a light chain distinct from those of the other members of the group.

To establish whether neutralizing ability could be related to antigen binding affinity, competition ELISAs were carried out in which soluble gp120 competed with gp120 coated on ELISA plates for Fab binding. Fig. 3 shows that there is no correlation between antigen affinity and neutralization. These experiments were carried out with gp120 of strain IIIB. All of the Fab fragments showed comparable ELISA reactivity with gp120 of strain SF2 (C.F.B. *et al.*, unpublished work).

To investigate whether neutralization could be associated with blocking of the gp120–CD4 interaction, competition ELISAs were carried out with soluble CD4 competing with Fabs for binding to gp120-coated ELISA wells. As shown in Fig. 4, CD4 inhibits the binding of neutralizing and nonneutralizing Fab fragments to gp120 comparably. This implies that blocking of the gp120–CD4 interaction is unlikely to be an important factor in Fab neutralization of HIV-1.

The ability of Fab fragments to neutralize viruses has been controversial. One of the problems has been that Fab fragments are classically generated by papain digestion of IgG. If the Fab, as is often the case, shows reduced activity relative to the parent IgG, then it may be difficult to rule out IgG contamination in the Fab preparation. A recent review concluded that "A clear answer to the question of the ability of monovalent Fab to neutralize virus has not emerged either" (1). By using recombinant Fab fragments we definitively show here that Fab fragments can neutralize virus.

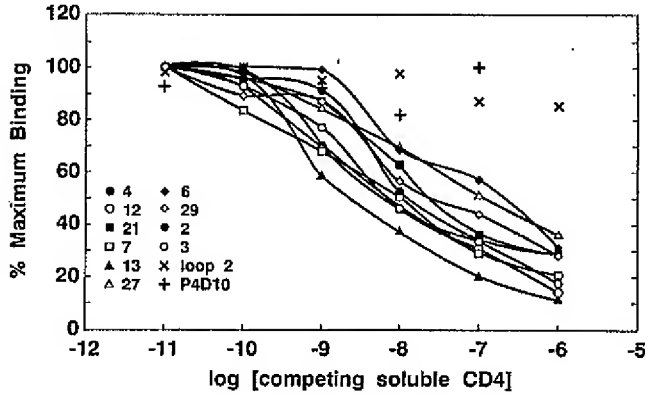

FIG. 4. Soluble CD4 competition with Fab fragments for gp120 (IIIB). P4D10 and loop 2 are controls not expected to compete with CD4. P4D10 is a mouse monoclonal antibody reacting with the V3 loop of gp120 (IIIB). As in Fig. 3, loop 2 Fab competition was carried out using gp120 (SF2).

The mechanism of neutralization of HIV-1 appears neither to require virion aggregation or gp120 crosslinking nor to be correlated with blocking of the gp120–CD4 interaction. One caveat here is that the experiments described refer to the interaction of monovalent CD4 and gp120 molecules, whereas the virus–cell interaction leading to infection is most likely cooperative and multivalent (37). Any importance attached to the difference will require studies on the relative abilities of the Fab fragments to affect viral attachment to a cell membrane. The existence of cloned neutralizing Fab fragments should allow the molecular features that confer neutralizing potential to be explored. For instance, in the case of the 13 group of clones (Fig. 2), the unique character of the light chain of the neutralizing clone (13) suggests that chain-shuffling experiments in which the 13 light chain is recombined with the other heavy chains in that group might be revealing. A previous report (37) showed how a heavy chain could be paired with two dissimilar light chains with retention of antigen affinity but altered fine specificity (26).

The observation here of a large number of Fab fragments of which only a limited number are strongly neutralizing may have important consequences. If the pattern is repeated for whole antibodies, then it would seem that much of the gp120 structure may be in a sense a "decoy"; i.e., the immune system may invest considerable effort in producing antibodies of high affinity but limited antiviral function. To exacerbate the situation, the ineffective antibodies may bind to gp120 and inhibit the binding of strongly neutralizing antibodies. This has obvious consequences for vaccination, which should be primarily designed to elicit such neutralizing antibodies. The determination of the precise molecular features responsible for eliciting protective neutralization then becomes an urgent priority.

We thank Richard A. Lerner and Robert M. Chanock for support and encouragement and Eva von Garrelts for technical assistance. We acknowledge the financial support of the National Institutes of Health, Johnson and Johnson, the U.S. Army Medical Research and Development Command (Grant DAMD-17-89-z-9038), The Swedish Medical Research Council, and The Swedish Cancer Society. C.F.B. is a Scholar of the American Foundation for AIDS Research.

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JOURNAL OF VIROLOGY, Jan. 2008, p. 1047–1052
0022-538X/08/$08.00+0 doi:10.1128/JVI.01986-07
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Vol. 82, No. 2

Identification of a Broadly Cross-Reacting and Neutralizing Human Monoclonal Antibody Directed against the Hepatitis C Virus E2 Protein[▽]

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Received 10 September 2007/Accepted 29 October 2007

Identification of anti-hepatitis C virus (anti-HCV) human antibody clones with broad neutralizing activity is important for a better understanding of the interplay between the virus and host and for the design of an effective passive immunotherapy and an effective vaccine. We report the identification of a human monoclonal Fab (e137) able to bind the HCV E2 glycoprotein of all HCV genotypes but genotype 5. The results of antibody competition assays and testing the reactivity to alanine mutant E2 proteins confirmed that the e137 epitope includes residues (T416, W420, W529, G530, and D535) highly conserved across all HCV genotypes. Fab e137 neutralized HCV pseudoparticles bearing genotype 1a, 1b, and 4 E1-E2 proteins and to a lesser extent, genotype 2b. Fab e137 was also able to inhibit cell culture-grown HCV (genotype 2a). These data indicate that broadly cross-reacting and cross-neutralizing antibodies are generated during HCV infection.

It is widely accepted that antibodies play a crucial role in the prevention and treatment of many viral infections of humans, including respiratory syncytial virus (16), rabies virus (34), and hepatitis B virus (35) infections. In contrast, a protective role of antibodies during infections by several persistent RNA viruses has not been widely accepted. In hepatitis C virus (HCV) infection, the frequent inability of the host to clear the virus and the possible reinfection after virus clearance (21) have been considered evidence against a protective role of specific antibodies. However, it has recently been shown that the anti-HCV antibody repertoire includes neutralizing and cross-reactive clones that are dispersed within a majority of antibody molecules that have minimal benefit for the host (8, 9, 25, 39, 36). Parallel analyses have recently suggested that antibodies play a crucial role in different phases of the natural history of HCV infection (3, 14, 15, 19, 30, 31).

In the present study, we characterized the anti-HCV E2 human monoclonal antibody (MAb) e137, which was cloned as a Fab fragment by phage display from the immunoglobulin G1 (IgG1) light-chain κ repertoire of an infected patient (7, 11). The E2-binding activity of Fab e137 is inhibited by sera of patients infected with different HCV genotypes (9, 25, 26), suggesting that this human MAb could recognize E2 proteins of a wide range of HCV genotypes and subtypes.

In order to better define the breadth of e137 cross-reactivity, we used human epithelial kidney (HEK) 293T cells expressing HCV E1-E2 of different genotypes (23). In detail, the HEK 293T cells were transfected with 3 μg of pcDNA3.1 vector (23), encoding E1-E2 glycoproteins from different HCV genotypes. The binding of e137 was assayed by immunofluorescence using a fluorescein isothiocyanate-conjugated anti-human Fab (Sigma) (18). Fab e137 was able to bind all HCV genotypes but genotype 5 (Fig. 1A). The data were confirmed using cells expressing HCV E1-E2 from other isolates (Fig. 1B). In only one case, e137 did not recognize HCV of genotype 2a (strain UKN2A2.4). The isolate UKN2A2.4 E2 sequence diverges by 17% from that derived from UKN2A1.2 (which was recognized by e137). These sequence differences likely cause a loss of contact residues or conformational changes that could make the epitope of e137 less accessible. The broad cross-reactivity of e137 was also confirmed by an immunoprecipitation assay performed on lysates of HEK 293 cells expressing E1-E2 glycoproteins from all genotypes (Fig. 1C). The immunoprecipitation assay was performed as previously described (28).

Considering these data, an important point is the definition of the HCV E2 regions having the potential of eliciting the cross-reactive antibody. Our previous attempts to identify the epitope recognized by e137 using multiple antigenic peptides of HCV envelope glycoprotein E2 were not successful (11). Furthermore, Fab e137 did not bind to recombinant maltose-binding protein–E2 fusion protein or to hypervariable region (HVR) multiple antigenic peptides using an enzyme-linked immunosorbent assay (ELISA) (data not shown). These data suggest that e137 is directed against a conformational epitope retained in the full-length HCV E2, as usually seen in broadly neutralizing antibodies (1, 5, 17, 18). Accordingly, as an alternative strategy for mapping the epitope recognized by e137, we used an ELISA competition assay with a panel of mouse and rat MAbs directed against known epitopes of genotype 1a HCV E2 (Table 1). Competition experiments were performed as described previously (5). Using this approach, binding of

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▽ Published ahead of print on 7 November 2007.

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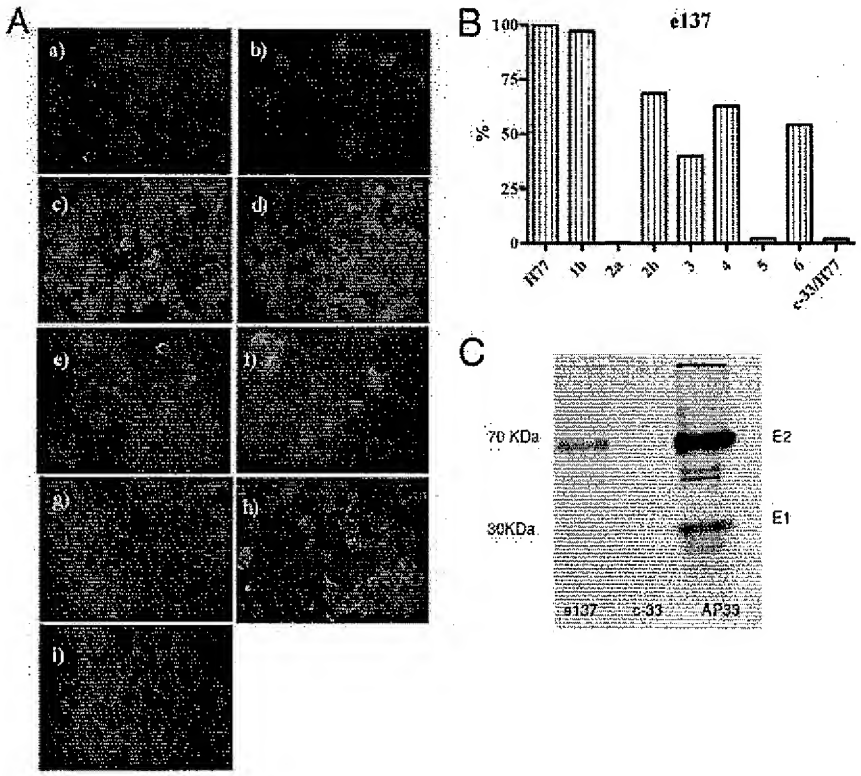

FIG. 1. (A) Analysis of binding of the Fab e137 by immunofluorescence staining of cells expressing E1-E2 proteins derived from different HCV genotypes. The cells were counterstained with Evans blue (red-stained cells). (a) Genotype 1a isolate UKN1A20.8; (b) genotype 1b isolate UKN1B5.23; (c) genotype 2a isolate UKN2A1.2; (d) genotype 2b isolate UKN2B1.1; (e) genotype 3 isolate UKN3A13.6; (f) genotype 4 isolate UKN4.21.16; (g) genotype 5 isolate UKN5.15.11; (h) genotype 6 isolate UKN6.5.8; (i) a human recombinant Fab (c33-3) specific for a nonstructural antigen of HCV (NS3) was included as a negative control (data generated on UKN1A20.8 are shown). Fab fragments were tested at a concentration of 10 μg/ml. (B) Binding activity of anti-HCV E2 Fab e137 on E1-E2 proteins derived from HCV isolates with different genotypes (genotypes 1a, 1b, 2a, 2b, 3, 4, 5, and 6): H77.20, UKN1B12.16, UKN2A.2.4, UKN2B2.8, UKN3A1.28c, UKN4.21.16, UKN5.15.11, and UKN6.5.8. Binding activity was expressed as a percentage of reactivity of the e137 Fab on E1-E2 proteins of genotype 1a (H77 strain). A human recombinant Fab (c33-3) specific for a nonstructural antigen of HCV (NS3) was included as a negative control (data generated on H77 are shown). The binding was assayed by fluorescence-activated cell sorting, using a fluorescein isothiocyanate-conjugated secondary anti-human Fab (Sigma) and measured by analysis of the percentage of cells with a higher fluorescence signal than cells without Fab. Fab e137 was also tested using untransfected cells, and this fluorescence was subtracted as background. The broadly cross-reactive AP33 was used in order to analyze the efficiency of transfection. The percentage of AP33-incubated cells with a higher fluorescence signal than untreated cells was at the same level among cells expressing E1-E2 proteins of different genotypes (data not shown). Fab e137 was tested at 10 μg/ml. (C) Radiolabeled proteins in the lysate of HEK 293T expressing E1-E2 glycoproteins of all genotypes were immunoprecipitated using e137. AP33 and c-33 were used as positive and negative controls, respectively. The immune complexes were analyzed by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis under reducing conditions. The protein sizes (in kilodaltons) are shown to the left of the gel. Data for E1-E2 of genotype 1a are shown.

e137 to HCV E2 was shown to be inhibited by the mouse MAb AP33 and two rat MAbs (2/64a and 9/75) each recognizing linear epitopes spanning E2 regions from amino acid (aa) 421 to 423, 524 to 531, and 528 to 535, respectively. Interestingly, the regions from aa 412 to 423 and 524 to 535 have been reported to be crucial for CD81 binding and retroviral pseudoparticle (HCVpp) infectivity (29). To confirm these data, we used a panel of H77-derived E1-E2 (genotype 1a) proteins containing alanine replacement mutants, some of which have been previously shown to be important for CD81 binding (29). The analysis of e137 binding of the panel of E1-E2 mutants (Fig. 2) confirmed that the e137 epitope is centered in aa 412 to 423 and aa 528 to 535 of HCV E2 regions, since substitutions at conserved positions 416, 420, 529, 530, and 535 reduced binding by greater than 90%. These data confirm that the conformational epitope bound by e137 includes conserved residues that are crucial for CD81 binding and HCVpp infectivity. These data are interesting, considering that e137 has been described to be an antibody with neutralization of binding activity (11). Furthermore, the data highlight that the epitope of e137 includes two conserved residues (aa 416 and 420) that were described to be critical within the epitope recognized by MAb AP33 (36). Interestingly, among the genotype 2a-derived E2 sequences studied in this paper (UKN2A1.2, UKN2A2.4, and JFH-1), a mutation from threonine to serine at position 416 was present only in the isolate not bound in the binding assay analyzed by fluorescence-activated cell sorting (UKN2A2.4), thus confirming that this mutation plays a crucial role in the lack of e137 binding to this strain. Indeed, T416 is quite conserved among different E2 genotypes, being always present in genotypes 1a, 1b, 2b, 3, 5, and 6. However, the T416S replacement has been reported in 59% of E2 sequences derived from genotype 2a and in 40% of E2 sequences derived from genotype 4 (37). As far as the other unbound genotype is concerned, all

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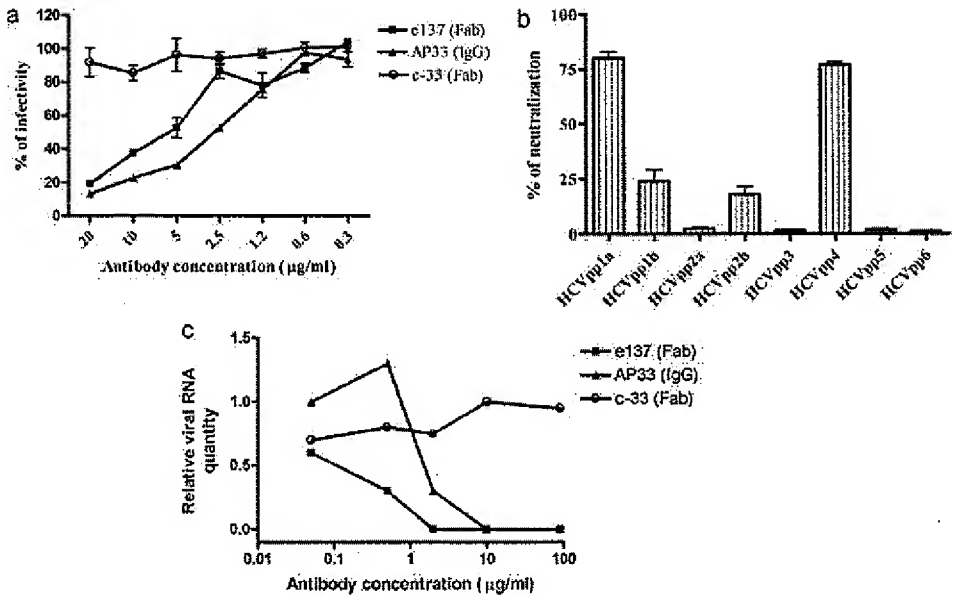

FIG. 3. (a) Neutralizing activity of Fab e137 using virus pseudoparticles displaying E1-E2 genotype 1a (UKN1A20.8). Data obtained from HCVpp infection in the presence of c33-3 (negative control [neg]), AP33, and in the presence of different concentrations (in micrograms per milliliter) of immunoaffinity-purified (>90%) Fab e137 are presented as a percentage of the infection detected in the absence of antibody. Neutralization activity was expressed by percent reduction of luciferase activity relative to the value for the control without competing antibodies. The experiment was performed three times, and the means ± standard errors of the means (error bars) from the three replicate assays are reported. (b) Neutralizing activity of Fab e137 at 15 μg/ml using virus pseudoparticles displaying E1-E2 proteins of different genotypes (HCV genotypes 1a to 6) (UKN1A20.8, UKN1B5.23, UKN2A1.2, UKN2B1.1, UKN3A13.6, UKN4.21.16, UKN5.15.11, and UKN6.5.8). The means plus standard errors of the means (error bars) of two replicate assays are reported. (c) Neutralization activity of e137 using the HCVcc system (genotype 2a). The infectivity of JFH-1 in the presence of e137, negative-control Fab (c33-3), and AP33 is presented as the viral RNA quantity normalized against glyceraldehyde-3-phosphate dehydrogenase RNA, as determined by quantitative reverse transcription-PCR. The virus infectivity was evaluated by measuring the levels of positive-stranded HCV RNA (24).

cross-reactive and cross-neutralizing human antibody clone generated during the natural course of HCV infection. This antibody is directed against a conformational epitope centered on the conserved HCV E2 regions from aa 412 to 420 and aa 528 to 535 and therefore outside hypervariable region 1 (HVR1). Importantly, the regions recognized by e137 show a lower variability rate than HVR1 does, and some E2 amino acid residues crucial for HCV infection are also critical for e137 binding. In particular, mutations of these residues generate variants able to escape from the e137 binding, but in parallel abrogate the infectivity of HCVpp (29). These data suggest that viral mutants able to escape e137 could have a reduced replication capacity. To date, the only MAb able to react with all HCV genotypes is AP33, a mouse MAb that is capable of potent neutralization of HCVpp representing a broad variety of HCV genotypes (28).Two intriguing points are that the epitope recognized by e137 partially overlaps with that of AP33 and that it is a broadly cross-neutralizing antibody in the pseudovirus-based neutralization assay. Indeed, e137 is able to neutralize HCVpp bearing E1-E2 of genotypes 1a, 1b, and 4 and to a lesser extent, genotype 2b. Moreover, e137 is able to neutralize HCVcc at a lower concentration than AP33 is. Notably, AP33 is a full-length immunoglobulin, while e137 is a Fab fragment, and the activity of a Fab molecule may increase in the whole immunoglobulin format (22, 38). Should the HCVcc neutralizing activity be a projection of the in vivo neutralizing potential and the IgG1 format increase the Fab neutralization activity by only 10-fold, a passive administration of e137-derived IgG MAb could easily reach serum levels potentially beneficial for the patient (2). Moreover, using e137 in combination with other neutralizing antibodies might result in an enhancement of the neutralizing activity and in a broadening of the panel of HCV genotypes neutralized.

Although several human MAbs against HCV have been described, the evidence of a broad cross-reactivity is still limited. Only a few anti-HCV E2 human MAbs have been shown to have cross-neutralizing activity. In particular, Fab 4, showed an IC_{50} from 0.3 to 10 μg/ml on HCVpp bearing E1-E2 of HCV genotypes 1a, 1b, and 2a, while data on HCVcc are not available (33). A group of anti-HCV IgG1 exhibited an IC_{50} ranging from 1.3 to 16 μg/ml and from 0.05 to 0.2 μg/ml, using HCVpp (bearing genotype 1b E1-E2) and the HCVcc system (genotype 2a), respectively (20); however, the antibodies were unable to neutralize HCV genotype 1a (27). Additionally, a recent clinical trial evaluated the use of a human MAb directed against HCV E2 as support in preventing the reinfection of patients with liver transplant for the end stage of the HCV liver disease (32). The trial showed an efficacy limited to the patients receiving very high doses; this could be due to the fact that the molecule used in this trial neutralizes HCVpp bearing E1-E2 of genotype 1a at 20 μg/ml (13), a dose difficult to reach in passive immunotherapy, leaving room for the expectation that a powerful antibody could possibly exert a beneficial effect in a similar clinical setting.

Overall, the availability of cross-reactive MAbs with

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strong neutralizing activity (i) allows a better understanding of the virus-host interplay, (ii) provides new opportunities to develop antigens potentially able to elicit a broadly neutralizing immune response, and (iii) may assist in the development of an effective passive immunotherapy for HCV infection.

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